EVOLUTION OF MATING SYSTEM AND INBREEDING DEPRESSION IN THE *MIMULUS MOSCHATUS* (SCROPHULARIACEAE) ALLIANCE

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A

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ABSTRACT

The transition from cross- to self-fertilization is considered a major pattern in the evolution of angiosperms. Yet, evolutionists continue to struggle to explain the evolutionary processes involved in maintaining both self- and cross-fertilization, which often occur within the same species. The diversity of mating systems suggests that selective pressures are also diverse, sometimes promoting selfing and other times promoting outcrossing. Inbreeding depression is commonly invoked as the primary selective force balancing the advantages of selfing or promoting outcrossing. The interaction between levels of inbreeding depression and mating system evolution has been fertile ground for both theoretical and empirical studies; however, long-term patterns and processes remain ambiguous.

I examined the relationship of inbreeding depression and outbreeding depression to mating system in a group of closely related *Mimulus* taxa, specifically incorporating information on their evolutionary relationships. I posed the following questions: Do selfing populations have low inbreeding depression and outcrossing populations have high outbreeding depression? Is selfing an evolutionary "dead-end"? Are morphological traits correlated with molecular estimates of mating system? How evolutionarily labile is mating system and inbreeding depression? Is inbreeding depression negatively correlated with outbreeding depression?

Results from this study largely supported theoretical expectations. Inbreeding depression was lowest in the most selfing species and highest in the most outcrossing species. Outbreeding depression was not observed. Many populations actually

experienced positive fitness consequences of between-population crosses. The question of selfing species being evolutionary dead-ends remained equivocal. Flower morphology was strongly related to molecular estimates of mating system as expected. Contrary to expectations, inbreeding depression appears to evolve much more quickly than does mating system.

I conclude that in the *Mimulus moschatus* alliance, inbreeding depression is not as strong a selective force as often implied in the evolution of mating system. Although generally low, inbreeding depression can be high in some populations of rare taxa. Outbreeding depression was minimal. Last, inbreeding depression was positively correlated with outbreeding depression, suggesting that mediating the negative effects of inbreeding depression cannot occur by the introduction of foreign genes for many populations.

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INTRODUCTION

One of the great mysteries in biology is the presence of an extremely broad diversity in organism form and function. Perhaps one of the most diverse and celebrated groups is the flowering plants. In particular, flowering plants display a cornucopia of pollination and mating systems. Within closely related groups (families, genera, or species) of plants, mating systems can range from obligate selfing, facultative selfing, facultative outcrossing, and obligate outcrossing species can coexist. While perhaps the complete range of mating systems is not often expressed in any particular plant group, generally a significant portion of them are. What can explain this diversity? Are there biological patterns within the general framework of this diversity, and what may explain them?

In some respects, the first question resides in the realm of metaphysics, and is beyond the scope of this investigation. However, we have made significant progress in addressing this question. Darwin's theory of evolution by natural selection posits the only strongly supported scientific theory for this diversity. Heritable variation arises naturally in populations, and natural selection can act on that variation. Often strong intraspecific selection exists, and traits that reduce competition with conspecifics are favored. Thus, diversity is promoted. However, this machinery producing diversity is running against those factors eliminating it (random genetic drift, extinction), and our current range of biodiversity is a balance of those forces.

For plant mating system diversity, a large number of selective forces favoring various mating systems have been proposed. The diversity of mating systems is evidence that no single selective agent is the primary force, and each population's characteristics are produced by a shifting balance of selective forces and random factors. One of the fundamental questions addressed in evolutionary biology is why more species are not complete selfers: a selfing mating system should be favored because double the number of gene copies are transmitted relative to outcrossing (Fisher 1941). However, if the fitness of offspring from selfing have reduced fitness (inbreeding depression), selection may counterbalance the gene transmission advantage, favoring outcrossing. The

interaction between the two counter-acting forces (gene transmission advantage and inbreeding depression) are generally regarded as the primary forces shaping mating-system evolution.

A widely accepted recurrent evolutionary pattern in flowering plants is the mating-system shift from outcrossing to selfing (Stebbins 1950, Grant 1981, Barrett et al. 1996). Theory predicts that selfing should evolve from outcrossing and not vice versa due to a number of combining factors (see review in Takebayashi and Morrell 2001). However, there have been very few explicit investigations into this pattern.

Additionally, numerous studies have improved our understanding of the relationship between inbreeding depression and mating system; however, an explicit evolutionary approach, combining data on phylogenetic relationships with estimated inbreeding depression and mating system values, is lacking.

To address these topics, I first explore the evolutionary relationships among species in the *Mimulus moschatus* alliance in Chapter 1. I construct a best-estimate phylogenetic tree, by combining both molecular and morphological data. Estimated pollination- and mating-system traits are mapped onto the phylogenetic tree to address whether outcrossing lineages give rise to selfers and not vice versa, and if selfing lineages are evolutionary dead-ends. In Chapter 2, I investigate correlations among mating-system traits in the study system and its sister clade, the *M. guttatus* complex. I ask whether morphological floral traits are closely associated with outcrossing rates, estimated from allozyme data. In Chapter 3, I describe the magnitude and timing of inbreeding depression in the study system and its relationship to mating system. I addressed three main points: 1) Is there an association between "fitness-conferring" and "selfing" alleles among populations and species? 2) Do taxa inherit a significant portion of both mating-system traits and inbreeding-depression levels from their ancestors? 3) What is the sequence of evolution of mating system and inbreeding depression? In Chapter 4, I tested the relationship among inbreeding depression, outbreeding depression, and mating system and discuss conservation implications based on the experimental results.

CHAPTER 1. PHYLOGENETIC RECONSTRUCTION, ECOLOGICAL EVOLUTION, AND BIOGEOGRAPHY OF THE *MIMULUS MOSCHATUS* ALLIANCE (SCROPHULARIACEAE)

ABSTRACT

To determine evolutionary patterns and test taxonomic hypotheses of biogeographic species associations, I conducted a phylogenetic analysis and reconstruction for species in the *Mimulus moschatus* alliance based on 52 characters. Life-history, vegetative, and reproductive traits were used to construct the phylogeny. The morphological phylogenies were compared with molecular phylogenies of nuclear ITS, ETS and chloroplast *rpl16*, *trnL* intron and exons (Whittall 1999, Beardsley unpubl.). A total evidence phylogeny was produced by synthesizing morphological and molecular data sets. By removing all morphological traits that could be associated with mating system, an "independent" phylogeny was constructed that could be further used to test hypotheses about mating-system evolution. Morphological phylogenetic patterns were largely consistent with molecular ones, although many of the relationships were equivocal. The lack of resolution was due partly to uncertainties in outgroup identity and to the large degree of homoplasy present in the morphological data. This indicated that many traits, including corolla size, perennial habit, and seed dormancy, are highly evolvable in this group. Ancestral-state reconstruction indicated annuals are derived from perennials, but two reversals to perennial habit have occurred. At least five transitions occurred in the evolution of seed dormancy, but the direction of transitions was largely equivocal. Pollination systems appeared to have shifted from large- to small-bee pollinated, as well as occasional subsequent transitions to autogamous pollination systems. No cases of autogamy giving rise to more outcrossing pollination systems were found, but one case of an autogamous ancestor giving rise to two autogamous species was indicated.

Widespread species appeared to have given rise to more geographically restricted species. Calyx, herbage, chromosome traits, and anther and pollen morphology appeared to be the most evolutionarily stable characters. The morphological data supported the presence of three biogeographic species associations (i.e., the Sierra Nevada, Columbia River, and Great Basin clades). The results also supported the taxonomic recognition of two previously synonomized species of conservation concern.

INTRODUCTION

Phylogenetic reconstructions can be used for generating and testing patterns of phenotypic, ecological, and biogeographic evolution (Mitter and Brooks 1983, Donoghue 1989, Brooks and McLennan 1991, Armbruster 1992, Bruneau 1997). Phylogenetic patterns have been used to identify geographic centers of diversity, when and where taxa evolved, and what events have led to the present geographical distribution (Olmstead 1989, Armbruster 1994, Trewick and Wallis 2001). These biogeographic investigations have also led to questions regarding the importance of ancestral migration in determining species patterns. Further, questions remain if widespread species that undergo range contractions play an important role in speciation, as hypothesized by Stebbins (1974). Additionally, past and present habitat conditions can have large effects on morphology and ecology. These habitat effects can also be reflected in phylogenetic patterns (Taylor and Hickey 1992). It is possible, for example, to investigate whether soil and seed germination requirements are evolutionarily conservative or labile, and thus whether these traits represent constraints in migration and distribution of plant taxa. The evolution of other life-history features offers fertile ground for examining hypotheses in a phylogenetic context. The perennial habit has been regarded as an ancestral trait, giving rise to annuals (Carlquist 1962, Stebbins 1974), although the universality of the pattern is questionable. Similarly, pollination systems have been shown to be evolutionary labile in some groups (Armbruster 1992, Bruneau 1997, Johnson et al. 1998, Barrett et al. 1997), but it is unknown how general these results may be. The development of computer-based

phylogenetic methods now make it possible to address such questions in a more rigorous manner (Donoghue 1989, Armbruster 1994).

In this study, results from morphological, cytological, and ecological data were used to infer the pattern of phylogenetic divergence and putative monophyly of the *Mimulus moschatus* Lindley alliance. The morphological phylogenetic reconstruction was compared with published molecular-based reconstructions, and a comprehensive supertree (sensu Sanderson et al. 1998) was constructed. The resulting supertree was then used to explore the evolution of pollination system, seed dormancy, biogeography and rarity, and habitat preference.

Mimulus L. (Scrophulariaceae) is a diverse genus of over 100 species, characterized by five-angled calyces, bilabiate corollas, bi-lobed thigmotropic stigmas, four didynamous stamens, and opposite leaves. The relationship of *Mimulus* to the rest of the family Scrophulariaceae (sensu lato) is uncertain. Recent molecular work (Olmstead et al. 2001) has suggested that the genus may be more closely related to the Asian genus, *Paulownia*, and some members of the *Lamiaceae* rather than the tribe Gratioleae (Scrophulariaceae) as suggested by Grant (1924). The center of the genus' distribution is in western North America, but a small number of species are present in South America, southern Africa and Madagascar, India, Australia, New Zealand, and eastern Asia. Grant (1924) recognized two subgenera, *Mimulus* and *Schizoplacus*, and ten sections. The two subgenera appear to be well supported by molecular analyses, and all sections are thought to represent natural groupings with the exception of section *Paradanthus*, which is likely a polyphyletic grouping of species complexes that may be allied with other sections in the genus (Grant 1924, Whittall 1999, Beardsley unpubl.). One group within section *Paradanthus* is the *Mimulus moschatus* Lindley alliance, which is believed to represent a natural clade. The *Mimulus moschatus* alliance (or complex - a term that is also been given to the wide array of morphologically distinct populations of the species *M. moschatus* and is thus avoided here) consists of 12 herbaceous species (Grant 1924, Argue 1980, 1986, Meinke 1983, 1992, 1995, Vickery 1995). These species share chromosome number (2n = 32; although *M. evanescens* is uncounted) and a unique

combination of viscid pubescence, reduced and equal calyx teeth, and acrescent fruiting pedicels (Grant 1924, Argue 1986, Meinke 1992). Illustrations of the *M. moschatus* alliance members and selected outgroups are presented in Appendix 1-1. Four species are widely distributed in the western Pacific states, while eight are regionally very restricted, some to a single river drainage (Fig. 1-1).

To achieve a historical perspective, and in a wider attempt to understand the evolution of the mating system and associated morphology (see Armbruster 1993, and review in Weller and Sakai 1999) of the *Mimulus moschatus* alliance, I explored the phylogenetic relationships among these 12 species. Additionally, the resulting trees were compared to existing trees, based on molecular data. The molecular phylogenetic estimations from Whittall (1999) and Beardsley (unpubl.) are presented in Figure 1-2. The cladistic analyses were performed using largely morphological data with additional chromosomal and ecological data. The morphological data included both vegetative and reproductive characters; and analyses were run separately on the "full evidence" data, and on the "independent" data, not likely associated with mating system. Because I examined the evolution of mating system characters, reproductive characters were excluded from the phylogenetic estimation to avoid circular reasoning (Armbruster 1992, but see Luckow and Bruneau 1997). Last, I developed a composite phylogenetic estimation, combining information from four published molecular phylogenies (based on two nuclear and two chloroplast gene sequences) and the morphological phylogeny presented here. The new composite phylogenetic estimation was used to examine monophyly of the *Mimulus moschatus* alliance, and regional clades defined from previous molecular phylogenies (Whittall 1999). In particular, I asked the question: are the "Columbia River," the "Snake River," the "Sierra Nevada," and the "Great Basin" clades (sensu Whittall 1999) supported by morphological evidence? The total-evidence and mating system-independent topologies were compared and a composite supertree (Bininda-Emonds and Sanderson 2001) was constructed based on all available phylogenies.

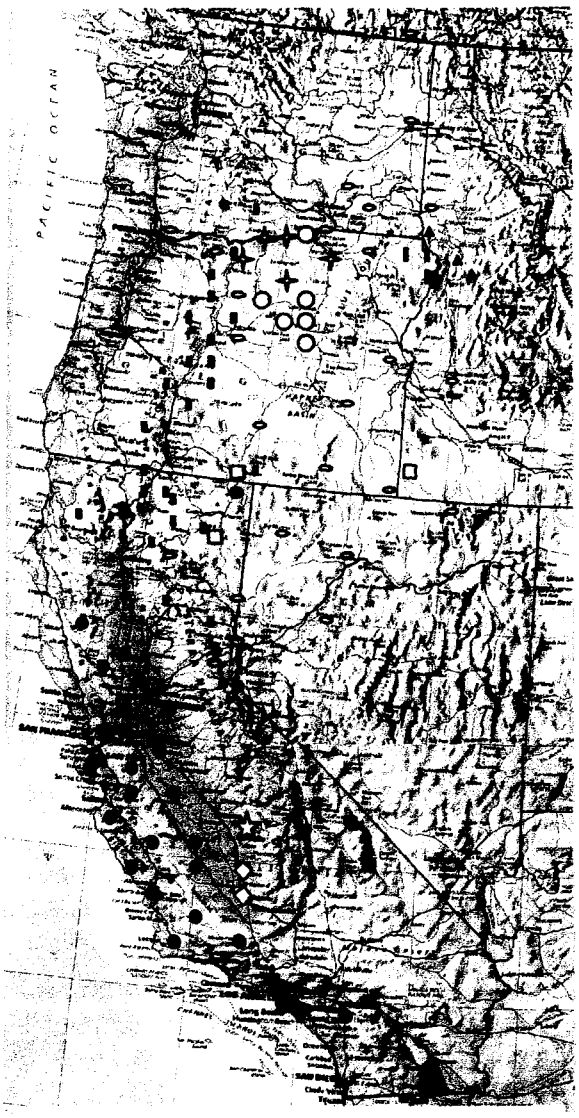


FIGURE 1-1. Distribution of the *Mimulus moschatus* Lindley alliance. ▲ = *M. ampliatus*, ⌓ = *M. breviflorus*, ◇ = *M. dudleyi*, □ = *M. evanescens*, ✤ = *M. hymenophyllus*, ✧ = *M. jungermannioides*, ● = *M. latidens*, ☆ = *M. norisii*, ▯ = *M. patulus*, ▮ = *M. pulsiferae*, ◯ = *M. washingtonensis*. The distribution of *M. floribundus* and *M. moschatus* are very widespread and are not shown here. *Mimulus floribundus* is found from southern British Columbia, east to South Dakota and south to New Mexico, with the greatest concentration of populations in the Sierra Nevada foothills. *Mimulus moschatus* ranges from British Columbia to the Rocky Mountains to western California.

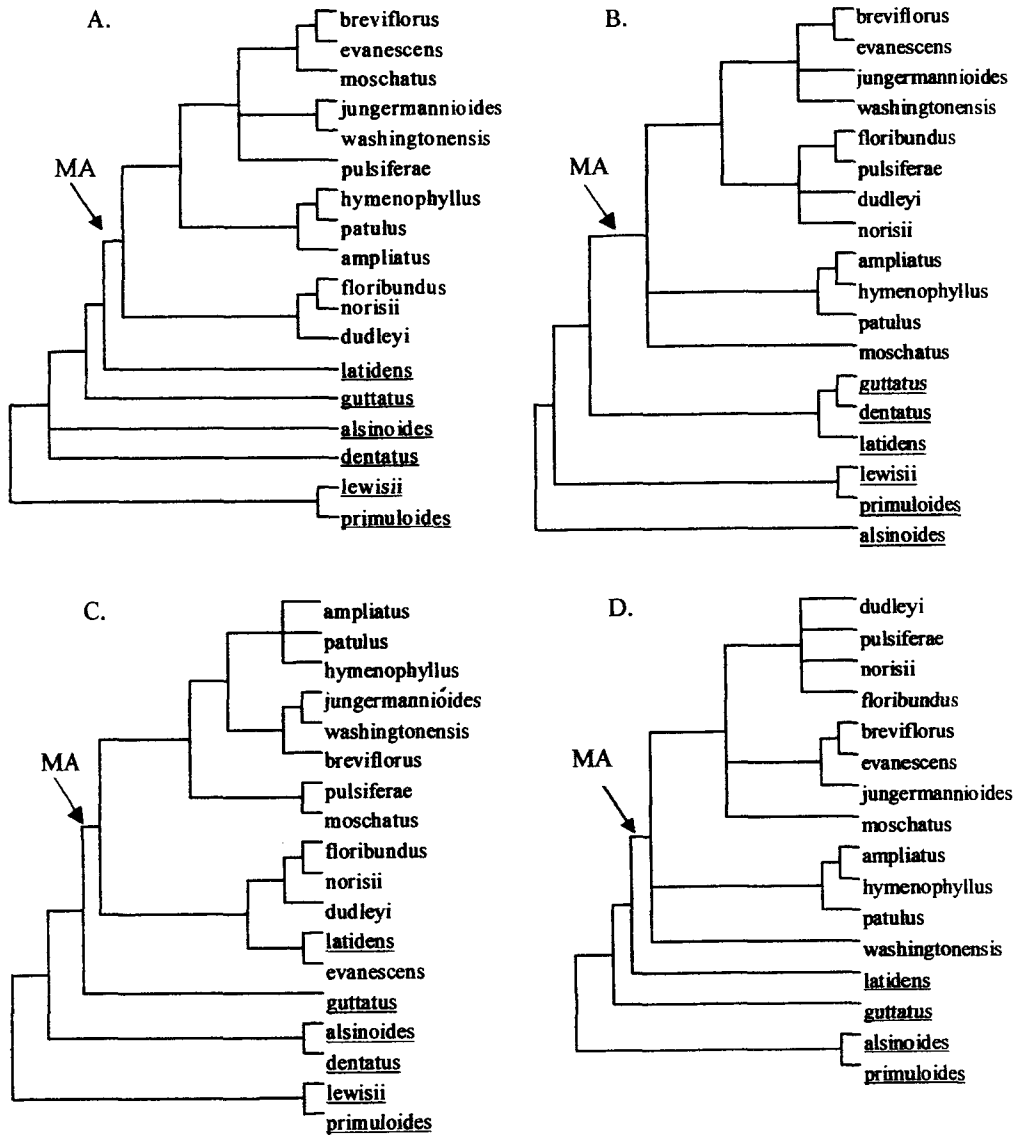


FIGURE 1-2. Molecular phylogenies of the *M. moschatus* alliance and various outgroups. A.) is the strict consensus tree ITS tree, B.) is the strict consensus *rpl16* tree (Whittall 1999). C.) is a combined ETS and ITS topology and D.) is a *trnL* topology (Beardsley unpubl.). MA = the *M. moschatus* alliance. Outgroups are underscored.

The resulting supertree was employed to explore the evolution of biogeographic patterns (including narrow endemism), substrate requirements, seed dormancy, perennial/annual habit, and primary pollination systems. Specifically, this study addresses the following questions: 1) Do phylogenetic and biogeographic patterns reflect widespread ancestral migration, or more conservative (in situ) speciation? 2) Do widespread species give rise to geographically restricted species, and is the restricted condition only a derived state? 3) Seed dormancy is present in roughly half the species in the *M. moschatus* alliance: are all the seed dormant species phylogenetically related, or is seed dormancy evolutionarily labile? 4) Perennial habit is generally viewed as a primitive state (Stebbins 1974); does this condition give rise irreversibly to annuals? 5) Last, these species and outgroups fall into three coarse pollination categories: large-bee pollinated, small-bee pollinated, and largely autogamously pollinated species. Are pollination systems as labile in this temperate clade as in other groups, e.g., the subtropical vine *Dalechampia* (Armbruster 1993) and the South African orchid *Disa* (Johnson et al. 1998)? 6) Does the evidence follow the generally accepted pattern of dead-end autogamous lineages (see review by Takebayashi and Morrell 2001), or is there evidence of autogamous species giving rise to more outcrossing bee-pollinated species?

METHODS

In the phylogenetic analysis, I included 52 chromosomal, ecological, vegetative, and reproductive characters (Table 1-1). Data were collected from living specimens at Oregon State University greenhouse, from herbarium specimens, and from published species descriptions. In selecting characters for this study, an attempt was made to inspect material from a number of populations to avoid population-specific character states. Without the knowledge of the genetic and developmental foundation of the traits, I can only offer hypotheses of primary homology. Chromosome numbers are based on previously published reports (Meinke 1992, Thompson 1993). Illustrations of vegetative and reproductive traits are included in Appendix 1-1.

TABLE 1-1. List of characters used in the cladistic analysis of the *M. moschatus* alliance. See Table 1-2 for character state distribution among species. * indicates characters used in the mating system-unbiased analysis.

LIFE-HISTORY, ECOLOGICAL, AND CHROMOSOME TRAITS: (Meinke 1983, Heckard & Shevock 1985, Meinke 1992, Thomson 1993, Meinke 1995, Vickery 1995).

1. *Longevity* (0=annual, 1=perennial).
2*. *Habit* (0=prostrate, 1=erect).
3*. *Runners or stolons* (0=absent, 1=present).
4*. *Seed dormancy* (0=absent, 1=present). This was tested for nearly all of the species by an 80 d period of cold stratification (20-100 seeds on moist paper at 4°C in the dark), followed by 21 d at room temperature and 16 h daylength.
5*. *Ploidy level* (1=diploid [n=8], 2=aneuploid [n=14, 15, 24, 28], 3=tetraploid [n=16]). *Mimulus lewisii* possess the ancestral chromosome number of eight, the ingroup are apparently ancient tetraploids. *Mimulus guttatus* has many chromosomal races.

VEGETATIVE TRAITS:

6*. *Stem architecture* (0=rarely or irregularly branched above the base, 1=branching progressively bifurcated).
7*. *Stem thickness* (0=thin, 1=wide and succulent).
8*. *Stem shape in cross-section* (0= more or less round, 1=obviously 4-angled).
9*. *Stem length* (0=much greater than pedicle length, 1=much less than pedicle length).
10*. *Basal leaf arrangement* (0=no persistent rosette, 1=persistent rosette). Some species retain a basal rosette into maturity.
11*. *Leaf venation* (0=3 or more, 1=only 3). Small or undeveloped leaves of all species often have three veins, but large, well-developed leaves of many species have five primary veins.
12*. *Upper leaf petiole* (0=petiolate, 1=leaves sessile).
13*. *Leaf petiole shape* (0=more or less round or ellipitical in cross-section, 1=winged).

14. Leaf base shape* (0=abruptly tapered, 1=gradually tapered).

15. Leaf margin* (0=entire to mildly toothed, 1=strongly toothed).

16. Herbage secretions* (0=not viscid, 1=viscid).

17. Fine-glandular herbage* (0=not finely glandular, 1=finely glandular). This character identifies glandular trichomes that are small (i.e., not producing a great amount of glandular secretions individually) and closely spaced.

18. Pubescence cell number* (0=single-celled, 1=multicelled).

19. Calyx pubescence* (0=uniform,1=not uniform)

20. Upper calyx tooth* (0=uniform in length with other teeth, 1=longer than other teeth).

21. Calyx lobes convergent in fruit* (0=not converging, 1=converging).

22. Lower calyx tooth* (0=uniform in length with other teeth, 1=longer than other teeth).

23. Calyx hairy* (0=not hairy, 1=noticeably hairy).

24. Calyx swollen* (0=not swollen, 1=swollen in fruit).

25. Calyx membranous* (0=not membranous, 1=membranous).

26. Calyx lobe length* (0=less than 2 mm in length, 1=greater than 2 mm in length).

27. Capsule insertion* (0=stalked, 1=sessile).

28. Capsule shape* (0=apex abruptly tapered, 1=apex mildly tapered).

REPRODUCTIVE TRAITS:

29. Seed reticulation* (0=not obvious under 20 x magnification, 1=obvious under 20 x magnification).

30. Seed color* (1=light tan to tan, 2=brown, 3=brownish-black).

31. Seed shape* (1=oblong to long elliptical, 2=ovoid to elliptical).

32. Corolla size (0=corolla not more than twice that of the calyx, 1=corolla more than twice that of the calyx).

33. Corolla color (1=lavender to purple, 2=yellow, 3=pale pink to white).

34. Corolla palate prominence (0=no or very small white patch, 1=prominent white patch).

35. Corolla shape (0=not obviously bilabiate, 1=strongly bilabiate).

36. *Corolla throat floor* (0=not deeply grooved, 1=deeply grooved).
37. *Exterior palate pubescence* (1=tapered, 2=clavate). This refers to the shape of pubescence found directly outside of the corolla tube, largely concentrated on the palate.
38. *Interior palate pubescence* (1=mammillate, 2=thick-mammilate, 3=fine-mammilate and clavate, 4=fine-mammilate). This characterizes the shape of pubescence found within the corolla tube.
39. *Interior palate ridges* (0=pubescent posteriorly, 1=glabrous posteriorly). While all species examined had interior palate pubescence, in some species the proximal portion of the palate ridges were glabrous.
40. *Corolla aperture* (0=constricted, 1=open). This character is relative to flower size: a large-flowered (15-25 mm in width) species would have a constricted aperture if the space at the corolla opening was 0-4.0 mm, while a small-flowered species (5-15 mm) would have a constricted aperture if the space at the corolla opening was 0-2.0 mm.
41. *Petal margins* (0=notched, 1=entire).
42. *Petal orientation* (0=not reflexed, 1=reflexed).
43. *Lateral petal orientation* (0=angled outwardly, 1=obviously angled downwardly).
44. *Lower-center petal size* (0=similar in size to other petals, 1=obviously larger than all other petals).
45. *Anther pubescence* (0=absent, 1=present).
46. *Anther opening* (1=opening widely to the point of being slightly reflexed, 2=opening widely but incompletely, 3=opened only at the apex).
47. *Anther sacs* (1=theca equal, 2=theca sub-equal).
48. *Pollen type* (1=IIb, 2=I, 3=IIc). For a detailed description of the pollen morphological types see Argue 1980, 1986.
49. *Stigma lobe shape* (1=obovate, 2=lanceolate, 3=ovate).
50. *Stigma lobe symmetry* (0=upper and lower lobes unequal, 1=upper and lower lobes equal).

51. *Stigma color* (1=white, 2=cream, 3=yellow).
52. *Style pubescence* (0=absent, 1=present). Pubescence on *M. guttatus* and *M. washingtonensis* is hispid and clearly evident under 10 x, while the style pubescence of *M. jungermannioides* is scabrous and evident under higher magnification. All other species have glabrous styles.

Terminal Taxa –

Character polarity was determined by outgroup comparison (Maddison et al. 1984, Watrous and Wheeler 1981, Olmstead 1989). A hierarchy of outgroups from within section *Paradanthus*, plus a member of section *Simiolus* Grant, and a member of the apparently more distant section *Erythranthe* were included to examine the monophyly of the *M. moschatus* alliance. The most closely related outgroup to the *M. moschatus* alliance is believed to be *M. latidens* (Meinke 1992, Whittall 1999).

Phylogenetic Analyses –

All phylogenetic analyses were conducted with PAUP* version 4b8 (Swofford 1998). For these analyses: maximum parsimony was used, characters were unordered and were given equal weights. TBR was the branch-swapping algorithm. Bootstrap support for each branch was determined with 100 replicates.

To test for an outgroup effect and examine regions of congruence among the ingroup taxa, I conducted multiple analyses, specifying different individual or sets of outgroups. In all analyses I included all the taxa that are believed to be the ingroup. These species include the following: *M. ampliatus, M. breviflorus, M. dudleyi, M evanescens, M. floribundus, M. hymenophyllus, M. jungermannioides, M. moschatus, M. norisii, M. patulus, M. pulsiferae,* and *M. washingtonensis*. In the full-outgroup model I included five additional taxa, including two from sections outside of *Paradanthus* (Grant 1924). In the nearest-outgroup model, only *M. latidens* was included as an outgroup. The identity of the nearest-outgroup is based on nuclear ITS data, suggesting *M. latidens* is sister to the ingroup (Whittall 1999). In the mixed-outgroup model, *M. latidens* and *M. guttatus* were both included. Finally, to explore congruence in relationships within the alliance, I reconstructed the phylogeny without outgroups.

Cladistic analyses were run on the full-character set and on the mating system-independent character set. The mating system-independent character set was a subset of

TABLE 1-2. Species by character state matrix used in the cladistic analysis of the *M. moschatus* alliance. Character numbers refer to those in Table 1-1. ? = unknown character states, * = mating system-unbiased characters. The upper six species are outgroups. *Mimulus lewisii* and *M. guttatus* are representatives from sections outside of *Paradanthus*.

species	1*	2*	3*	4*	5*	6*	7*	8*	9*	10*
M. lewisii	1	1	0	?	1	0	1	0	0	0
M. guttatus	(0,1)	1	0	(0,1)	(2,3)	0	1	1	0	0
M. alsinoides	0	1	0	0	?	1	0	0	0	0
M. dentatus	1	(0,1)	0	0	?	1	0	0	0	0
M. primuloides	1	0	1	?	?	1	0	0	1	1
M. latidens	0	1	0	1	?	1	0	0	0	1
M. ampliatus	0	1	0	1	?	1	0	1	0	0
M. breviflorus	0	1	0	1	?	1	0	0	0	0
M. dudleyi	0	1	0	0	?	1	0	0	0	0
M. evanescens	0	1	0	1	?	1	0	0	0	0
M. floribundus	0	(0,1)	0	0	3	1	0	0	0	0
M. hymenophyllus	0	0	0	1	3	1	0	0	0	0
M. jungermannioides	1	0	1	0	3	1	0	0	0	0
M. moschatus	1	(0,1)	0	0	3	1	0	0	0	0
M. norisii	0	1	0	?	3	1	0	0	0	0
M. patulus	0	1	0	1	3	1	0	0	0	0
M. pulsiferae	0	1	0	1	3	1	0	0	0	1
M. washingtonensis	0	1	0	1	3	1	0	0	0	0

species	11*	12*	13*	14*	15*	16*	17*	18*	19*	20*
M. lewisii	0	1	0	1	1	0	0	1	1	0
M. guttatus	0	1	0	0	1	0	0	0	?	1
M. alsinoides	0	0	1	0	1	1	1	0	1	0
M. dentatus	0	1	0	1	1	0	0	1	1	0
M. primuloides	1	1	0	1	0	0	0	1	0	0
M. latidens	0	1	0	1	0	0	0	0	1	0
M. ampliatus	0	0	0	0	0	1	1	0	1	0
M. breviflorus	1	0	0	1	0	1	1	0	1	0
M. dudleyi	0	0	0	1	1	1	0	1	0	0
M. evanescens	0	1	0	1	0	1	1	0	1	0
M. floribundus	0	0	0	0	1	1	0	1	0	0
M. hymenophyllus	0	0	0	0	1	1	1	0	1	0
M. jungermannioides	0	0	0	0	1	1	1	(0,1)	0	0
M. moschatus	0	1	0	1	1	1	0	1	0	0
M. norisii	0	0	0	1	0	1	0	1	0	0
M. patulus	0	0	0	0	0	1	1	0	1	0
M. pulsiferae	1	0	0	1	0	1	1	0	1	0
M. washingtonensis	0	0	0	0	0	1	1	0	1	0

species	21*	22*	23*	24*	25*	26*	27*	28*	29*	30*
M. lewisii	0	0	1	0	0	1	1	0	1	2
M. guttatus	1	0	0	1	1	1	0	0	1	2
M. alsinoides	0	1	0	0	0	0	1	1	0	2
M. dentatus	0	0	1	0	0	1	1	1	0	2
M. primuloides	0	0	0	0	0	0	1	1	0	2
M. latidens	1	0	0	1	1	0	0	0	0	2
M. ampliatus	0	0	0	0	0	0	1	1	0	2
M. breviflorus	0	0	0	1	0	0	1	1	0	2
M. dudleyi	0	0	1	0	0	0	1	1	0	1
M. evanescens	1	0	0	1	1	0	1	0	0	2
M. floribundus	0	0	1	0	0	0	1	1	0	1
M. hymenophyllus	0	0	0	0	0	0	1	0	0	3
M. jungermannioides	1	0	1	1	0	0	1	1	0	2
M. moschatus	0	0	1	0	0	1	1	1	0	2
M. norisii	1	0	1	0	0	1	1	?	0	1
M. patulus	0	0	0	0	0	0	1	1	0	2
M. pulsiferae	0	0	0	0	0	0	1	1	0	2
M. washingtonensis	0	0	0	0	0	0	1	1	0	2

species	31*	32	33	34	35	36	37	38	39	40
M. lewisii	1	1	1	0	1	1	1	1	0	1
M. guttatus	1	(0,1)	2	1	1	0	1	1	0	0
M. alsinoides	1	1	2	1	1	0	1	4	0	1
M. dentatus	1	1	2	0	1	1	2	3	1	1
M. primuloides	1	1	2	1	0	0	2	2	0	1
M. latidens	1	0	3	?	0	0	2	1	0	1
M. ampliatus	1	1	2	0	1	0	2	1	0	1
M. breviflorus	1	0	2	0	0	0	?	?	?	1
M. dudleyi	2	1	2	0	0	1	2	1	1	1
M. evanescens	1	0	2	0	0	0	2	?	?	1
M. floribundus	2	0	2	0	0	0	2	1	1	1
M. hymenophyllus	1	1	2	0	1	0	2	1	0	1
M. jungermannioides	1	1	2	1	1	0	2	1	0	0
M. moschatus	1	1	2	0	0	1	1	1	0	1
M. norisii	2	1	2	1	0	?	2	1	1	1
M. patulus	1	0	2	0	0	0	2	1	0	1
M. pulsiferae	1	(0,1)	2	0	0	0	2	1	0	1
M. washingtonensis	1	1	2	1	1	0	2	4	0	0

species	41	42	43	44	45	46	47	48	49	50
M. lewisii	0	1	1	0	1	1	1	1	2	0
M. guttatus	0	1	1	1	0	3	1	2	1	1
M. alsinoides	1	0	1	0	0	2	1	1	2	0
M. dentatus	0	1	1	0	1	1	1	3	2	1
M. primuloides	0	0	0	0	1	2	?	2	2	1
M. latidens	1	0	0	0	0	2	?	3	3	0
M. ampliatus	0	1	0	0	0	2	2	?	3	1
M. breviflorus	?	0	0	0	0	?	?	3	1	?
M. dudleyi	1	0	0	0	0	1	1	?	1	1
M. evanescens	1	0	0	0	0	?	?	?	?	1
M. floribundus	1	0	0	0	0	1	1	1	1	1
M. hymenophyllus	0	1	0	0	0	2	2	3	2	1
M. jungermannioides	0	1	1	0	0	2	2	3	2	0
M. moschatus	1	0	0	0	1	1	1	3	?	?
M. norisii	1	0	0	0	0	?	?	1	1	1
M. patulus	1	0	0	0	0	2	2	3	2	1
M. pulsiferae	1	0	0	0	0	2	2	1	1	1
M. washingtonensis	0	1	1	0	0	2	2	3	1	1

species	51	52
M. lewisii	?	1
M. guttatus	?	1
M. alsinoides	1	0
M. dentatus	1	0
M. primuloides	?	0
M. latidens	?	0
M. ampliatus	2	0
M. breviflorus	?	0
M. dudleyi	1	0
M. evanescens	1	0
M. floribundus	1	0
M. hymenophyllus	2	0
M. jungermannioides	2	1
M. moschatus	1	0
M. norisii	?	0
M. patulus	2	0
M. pulsiferae	?	0
M. washingtonensis	3	1

the full-set, composed of 30 of the original 52 characters, including all vegetative characters and those not likely related to pollination or selective pressures directly associated with mating system. For each analysis the strict consensus tree and a single, randomly selected, most parsimonious tree (with branch lengths) are presented.

Last, a composite approach was taken to synthesize disparate sources of phylogenetic information into a single tree, using matrix representation with maximum parsimony in the construction. Supertrees combine the positive aspects of total evidence (sensu Kluge 1989) and taxonomic congruence (sensu Mickevich 1978) to avoid each method's drawbacks (Bininda-Emonds and Sanderson 2001). Like taxonomic congruence, supertree construction uses actual tree topology, and therefore avoids the potential pitfalls of data set incompatibility. Additionally, like total evidence, supertree reconstruction can combine estimates for different groups of terminal taxa, obtaining resolutions not present in any single previous source. The procedure of supertree construction (here, using matrix representation with parsimony analysis) involves coding the topology of each tree included in the analysis as a series of binary elements, one for each node in the tree. If taxa share a given node they are scored as "1"; if they do not share the node but are still present on the tree, they are scored as "0"; all other taxa are scored as "?". The matrix representations for all trees are combined into a single matrix. Parsimony analysis then is used to analyze the matrix. Supertree use shows great utility for phylogenetic inference by using all available information (Bininda-Emonds and Sanderson 2001).

Initial data sources originate from five tree topologies: 1) Beardsley (unpubl.), based on *trn*L intron and intergenic spacers between *trn*L and *trn*F; 2) Beardsley (unpubl.), based on combined nuclear ITS and ETS data; 3) Whittall (1999), based on chloroplast *rpl16* intron; 4) Whittall (1999), based on combined nuclear ITS1 and ITS2 regions; and 5) from the full and independent morphological data sets presented here. Each of these data sets had different species included and possessed very different types of data, favoring supertree construction over a combined data approach.

Evolution of ecological states –

The resulting independent supertree was used to investigate the evolution of biogeographic patterns, rarity, substrate requirement, seed dormancy, perennial habit, and primary pollination system using ancestral reconstructions in MacClade version 3.08 (Maddison and Maddison 1992). Biogeographic and ecological states for each species are given in Table 1-3. Biogeographic range was divided into six categories: 1) widespread low-mid elevation western North America, 2) widespread montane western North America, 3) Great Basin, 4) Columbia River Plateau, 5) Snake River Canyon (specifically, Hell's Canyon section), and 6) southern Sierra Nevada Foothills. Geographically restricted species (i.e., narrow endemics) were defined based on distributions of less than 300 km between the most distant populations (see Fig. 1-1). This designation captures all species that have been formally listed as Threatened, Endangered, or Species of Concern.

Substrate type was classified by the primary soil-type in which the species is typically found. Three substrate classifications are assigned based on personal observation, these include: 1) rich-organic soils, 2) poor-organic gravels (typically basalt), and 3) cliff-faces or rocky outcrops. *Mimulus guttatus* commonly inhabits all three substrate types and was therefore coded as polymorphic.

Presence or absence of winter seed dormancy was determined by either previous publications (Meinke 1992) or communications (R. J. Meinke), or by subjecting 20-100 seeds per species to two germination treatments. Half the seeds per species were placed on moist filter paper at room temperature and 16 h daylength; the other half were subjected to 80 d of cold stratification (on moist filter paper under continual dark at 4°C) followed by 21 d at room temperature and 16 h daylength. *Mimulus ampliatus*, *M. evanescens*, *M. hymenophyllus*, and *M. patulus* typically require cold stratification, but a very small percentage (ca. 1 %) of seeds will germinate without stratification (pers. obs.). These species were all classified as seed dormant.

TABLE 1-3. Species by biogeographic and ecological character state in the *M. moschatus* alliance. The upper six species are outgroups, *Mimulus lewisii* and *M. guttatus* are representatives from sections outside of *Paradanthus*. See text for a more detailed description of character states.

species	geographic category	narrow endemism	soil type	seed dormancy	perennial	Pollination system
M. lewisii	widespread montane	no	organic	equivocal	yes	large-bee
M. guttatus	widespread lowland	no	equivocal	no	yes/ equivocal	large-bee/ equivocal
M. alsinoides	widespread montane	no	cliff	no	no	small-bee
M. dentatus	widespread montane	no	organic	no	yes	large-bee
M. primuloides	widespread montane	no	organic	yes	yes	small-bee
M. latidens	widespread lowland	no	inorganic	no	no	autogamous
M. ampliatus	Snake River Canyon	yes	inorganic	yes	no	small-bee
M. breviflorus	Great Basin	no	inorganic	yes	no	autogamous
M. dudleyi	Sierra Nevada foothills	yes	cliff	no	no	small-bee
M. evanescens	Great Basin	yes	inorganic	yes	no	autogamous
M. floribundus	widespread lowland	no	inorganic	no	no	autogamous
M. hymenophyllus	Snake River Canyon	yes	cliff	yes	no	small-bee
M. jungermannioides	Columbia River Plateau	yes	cliff	no	yes	small-bee
M. moschatus	widespread montane	no	organic	no	yes	small-bee
M. norisii	Sierra Nevada Foothills	yes	cliff	no	no	small-bee
M. patulus	Snake River Canyon	yes	inorganic	yes	no	autogamous
M. pulsiferae	widespread montane	no	inorganic	yes	no	small-bee
M. washingtonensis	Columbia River Plateau	yes	inorganic	no	no	small-bee

The evolution of perennial/annual habit was investigated to determine if the perennial state is ancestral and if this trait is evolutionarily labile or conservative. Two species in the ingroup are perennials. *Mimulus moschatus*, like most of the perennial outgroups, re-sprouts from underground rhizomes, while *M. jungermannioides* re-sprouts from unique bulb-like structures (turions). The outgroup, *M. guttatus*, has both annual and perennial forms and was coded first as perennial, and then polymorphic. The character state assignment of this species did not significantly alter evolutionary reconstructions.

Pollination system was divided into three classes: 1) autogamous, 2) small-bee pollinated, and 3) large-bee pollinated. Assignment to classes was based on personal observation, published accounts (Kiang 1972, Meinke 1992, Bradshaw et al. 1995), and corolla morphology. Pollination system was classified as autogamous if corolla sizes were minute (< 5 mm in diameter) and nearly cleistogamous with little or no anther-stigma separation. Small-bee pollinated species are those with moderately sized corollas, which are narrow enough to exclude bumblebees. Meinke (1992) and I have observed only small (< 12 mm long) solitary bees primarily of the genera *Andrena*, *Ceratina*, *Lasioglossum* sensu lato, and *Osmia* visiting the species classified as small-bee pollinated. Large-bee pollinated species are those with corollas greater than 20 mm in diameter that also have accounts of bumblebees (*Bombus* spp.) as the primary visitors (Kiang 1972, Bradshaw et al. 1995). These three subdivisions are obviously coarse; for example, "large-bee" pollinated species undoubtedly receive visitation by smaller bees in some years and locations, and all species are capable of some seed set in the absence of visitation (see Dole 1990, Carlson unpubl. manuscript). Additionally, two of the smaller-flowered (autogamous) species have some populations with larger flowers that would be classified as small-bee pollinated under these criteria. I therefore coded these species under both classifications. *Mimulus guttatus* is a highly plastic species with populations of divergent corolla sizes, and thus variable pollination systems. Due to this variation, I conducted analyses in two ways: assuming large-bee pollination for *M. guttatus* sensu stricto, and undefined for *M. guttatus* sensu lato. To examine the evolutionary flexibility

of pollination system relative to the morphological characters used to construct the phylogeny, Armbruster's (1992) method of comparing consistency indices was used to compare pollination system to the average mating-system independent characters. It can be assumed that pollination system is more homoplastic than mating-system independent morphological traits if the consistency index for pollination system is much lower than the independent morphological characters.

Reconstructions were performed using unordered, equal weighting of character states in MacClade version 3.08 (Maddison and Maddison 1992). All figures are displayed with unordered character states; however, to visualize extremes in evolutionary patterns due to traits being evolutionarily conservative or labile, accelerated- and delayed-transformation options were employed. Additionally, the equivocal cycling procedure was used to explore all possible transitions. Under these option polytomies must be resolved into a series of dichotomies. Therefore the Snake River trichotomy was resolved in the two topologies observed in molecular trees: *M. patulus* + *M. hymenophyllus* sister to *M. ampliatus*, and *M. ampliatus* + *M. hymenophyllus* sister to *M. patulus*. The outgroup polytomies were resolved into a basal grade.

RESULTS

The full data set estimate –

The unconstrained data set contained 52 characters and 18 species, six of which were outgroups. A total of 133 maximally-parsimonious trees were obtained, each of 131 steps (CI=0.46, RI=59). Clades resolved in the strict consensus of all most parsimonious trees included *M. latidens* + *M. evanescens*; and one composed of *M. floribundus* + *M. dudleyi* + *M. norisii* (i.e., the Sierra Nevada Clade, sensu Whittall 1999). A single randomly selected most parsimonious tree and the strict consensus tree are presented in Figure 1-3. An additional clade revealed by the analysis includes a broad "*M. washingtonensis* alliance": *M. latidens* + *M. evanescens*, *M. pulsiferae*, *M. patulus*, *M. ampliatus*, *M. hymenophyllus*, *M. washingtonensis*, *M. jungermannioides*, *M. breviflorus*,

and *M. alsinoides*. The *M. moschatus* alliance was resolved, composed of the *M. washingtonensis* alliance plus *M. primuloides*, and the Sierra Nevada clade. It is important to note three species specified as outgroups could not be rooted outside of the *M. moschatus* alliance. The three species are from sect. *Paradanthus* (as the ingroup): *M. latidens*, *M. alsinoides*, found within the *M. washingtonensis* alliance, and *M. primuloides*, found within the *M. moschatus* alliance. All of these three species had very long branches (Fig. 1-3A). The two outgroups from separate sections (*M. lewisii* and *M. guttatus*), and *M. dentatus* of sect. *Paradanthus*, were well supported outside of the *M. moschatus* alliance.

Outgroup identity/inclusion seriously affected ingroup topology. When only the nearest proposed outgroup (*M. latidens*) was included, better resolution within the *M. moschatus* alliance was achieved. A total of 38 most parsimonious trees were obtained, each of 72 steps (CI = 0.64, RI =0.68). Not surprisingly, *M. latidens* and its sister, *M. evanescens*, were moved from a highly derived position within the *M. moschatus* alliance to a basal position (Fig. 1-4 A. and B.). Within the ingroup, *M. moschatus* was included with the Sierra Nevada clade; the Columbia River clade was well supported and was rooted in a broader clade that included the Snake River clade. *Mimulus pulsiferae* and *M. breviflorus* were located basally in the highly supported *M. moschatus* alliance.

When the next distant outgroup (i.e., *M. guttatus*) was used in place of *M. latidens*, some of the well supported clades disappeared (Fig. 1-4 C. and D.). A total of 54 most parsimonious trees were found of 83 steps (CI=0.63, RI=0.65). The Sierra Nevada clade + *M. moschatus* continued to be well supported. The remaining positions were less certain; the members of the Snake River clade and Columbia River clade tended to cluster together, but very weakly. With the inclusion of *M. guttatus* as the outgroup, one notable change in tree topology was the position of *M. jungermannioides*.

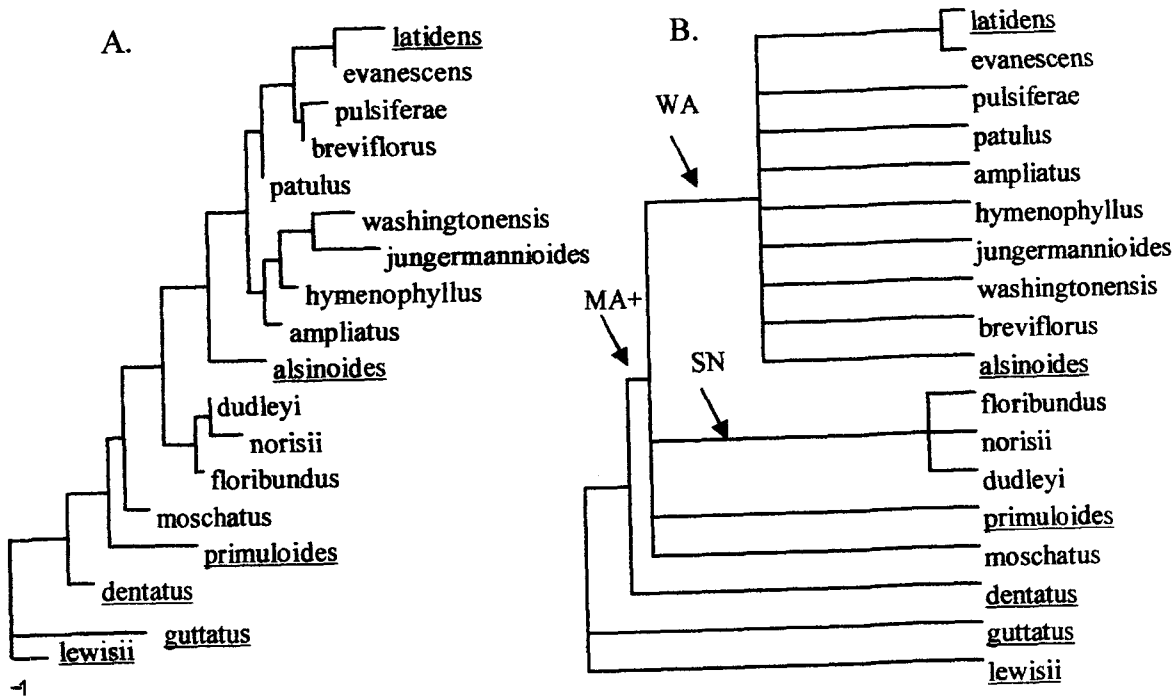


FIGURE 1-3. Phylogenetic reconstructions for the *Mimulus moschatus* alliance with the full morphological data set and all outgroups included. A.) is one of 131 randomly selected most parsimonious phylogenetic trees showing branch lengths and B.) is the strict consensus tree. Outgroups are indicated by underlining. WA = *M. washingtonensis* alliance, MA + = *M. moschatus* alliance plus three additional outgroups, SN = Sierra Nevada clade.

This species was pulled down into a basal location and its close relative, *M. washingtonensis*, followed. While *M. jungermannioides* and the highly plastic *M. guttatus* share many characters (perennial, swollen calyx in fruit, convergent calyx lobes, toothed leaf margin, etc.), these two species are from different sections, and clearly many of these shared traits are not homologous.

When both *M latidens* and *M guttatus* were included as outgroups, the species relationships remain poorly resolved (Fig. 1-5 A. and B.). Fifty-seven most parsimonious trees of 90 steps were obtained (CI=0.59, RI=0.64). The strict consensus tree resolved *M. latidens* and *M. evanescens* as a monophyletic group, and the Sierra Nevada clade + *M. moschatus* was resolved as a monophyletic assemblage. *Mimulus pulsiferae* and *M. breviflorus* tended to be allied with the previously mentioned two groups. The Snake River assemblage formed a paraphyletic grade basal to the aforementioned species. *Mimulus washingtonensis* was sister to the outgroup plus *M. jungermannioides*, a species which could not be rooted within the ingroup.

Relationships within the *M. moschatus* alliance were reasonably well resolved when no outgroups were included (Fig. 1-5 C. and D.) and were similar to topologies with *M. latidens* as the nearest outgroup (see Fig. 1-4 A. and B.). Thirty-eight most parsimonious trees of 65 steps were obtained (CI=0.66, RI=0.69). The strict consensus tree resolved the Sierra Nevada clade and the Sierra Nevada clade + *M. moschatus*. The Columbia River clade was also well supported, and to this group, the Snake River assemblage formed a basal grade. The major source of ambiguity surrounded the placement of two of the smaller flowered species, *M. breviflorus* and *M. pulsiferae*. *Mimulus evanescens* tended to be rooted basally.

The mating system-independent estimate –

Since the wider goal of this research is to explore the evolution of mating systems, I constructed phylogenies based on a subset of data composed of characters that are likely not involved in pollination or reproduction. This reduces the hazards of circularity in

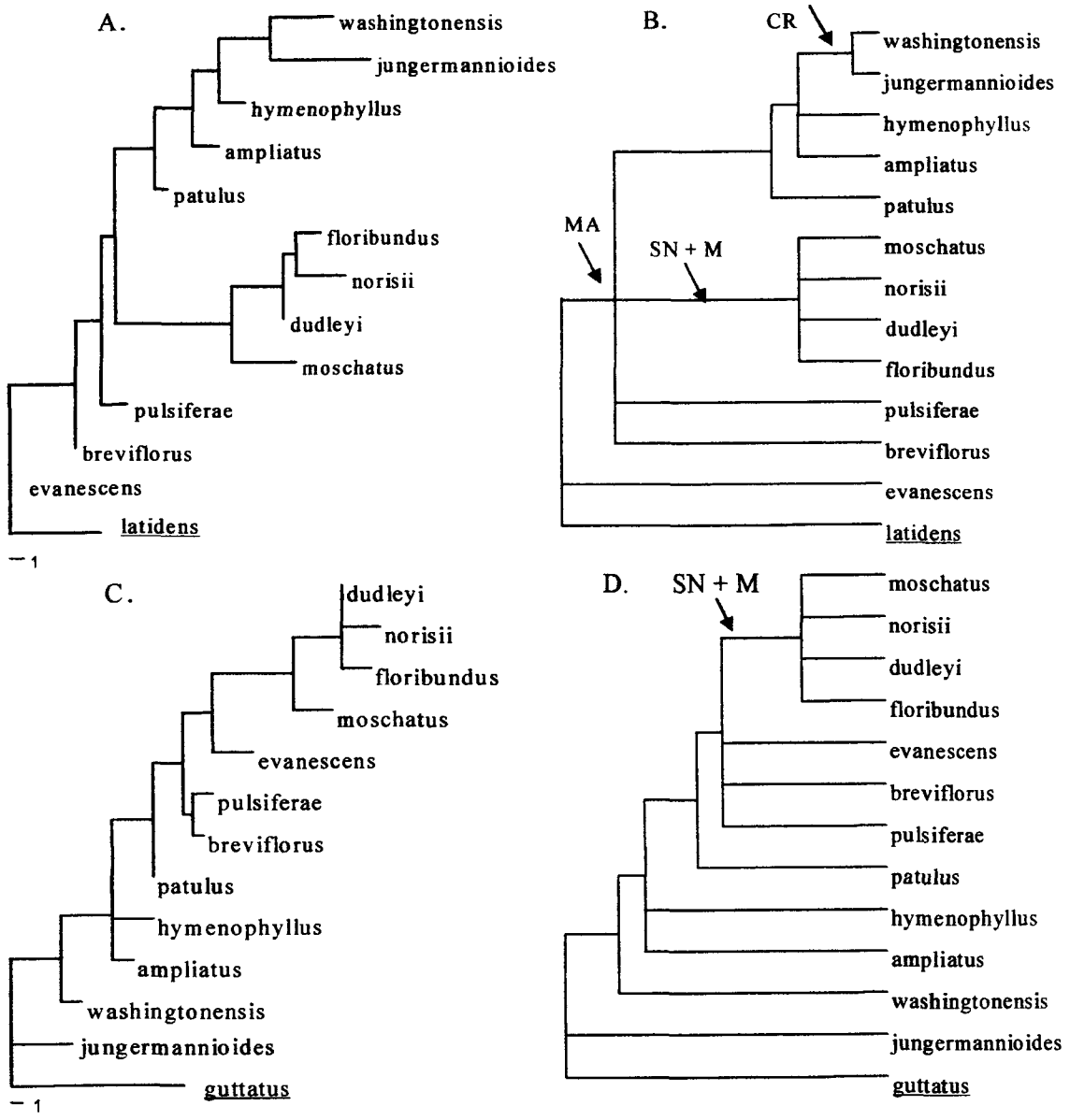


FIGURE 1-4. Phylogenetic reconstructions of the *M. moschatus* alliance with the full morphological data set and two different outgroups. A.) is one of 38 randomly selected most parsimonious phylogenetic trees showing branch lengths and B.) is the strict consensus tree when *M. latidens* is the only outgroup. C.) is one of 54 randomly selected most parsimonious trees and D.) is the strict consensus tree when *M. guttatus* is the only outgroup. CR = Columbia River clade, SN + M = Sierra Nevada + *M. moschatus* clade, MA = *M. moschatus* alliance.

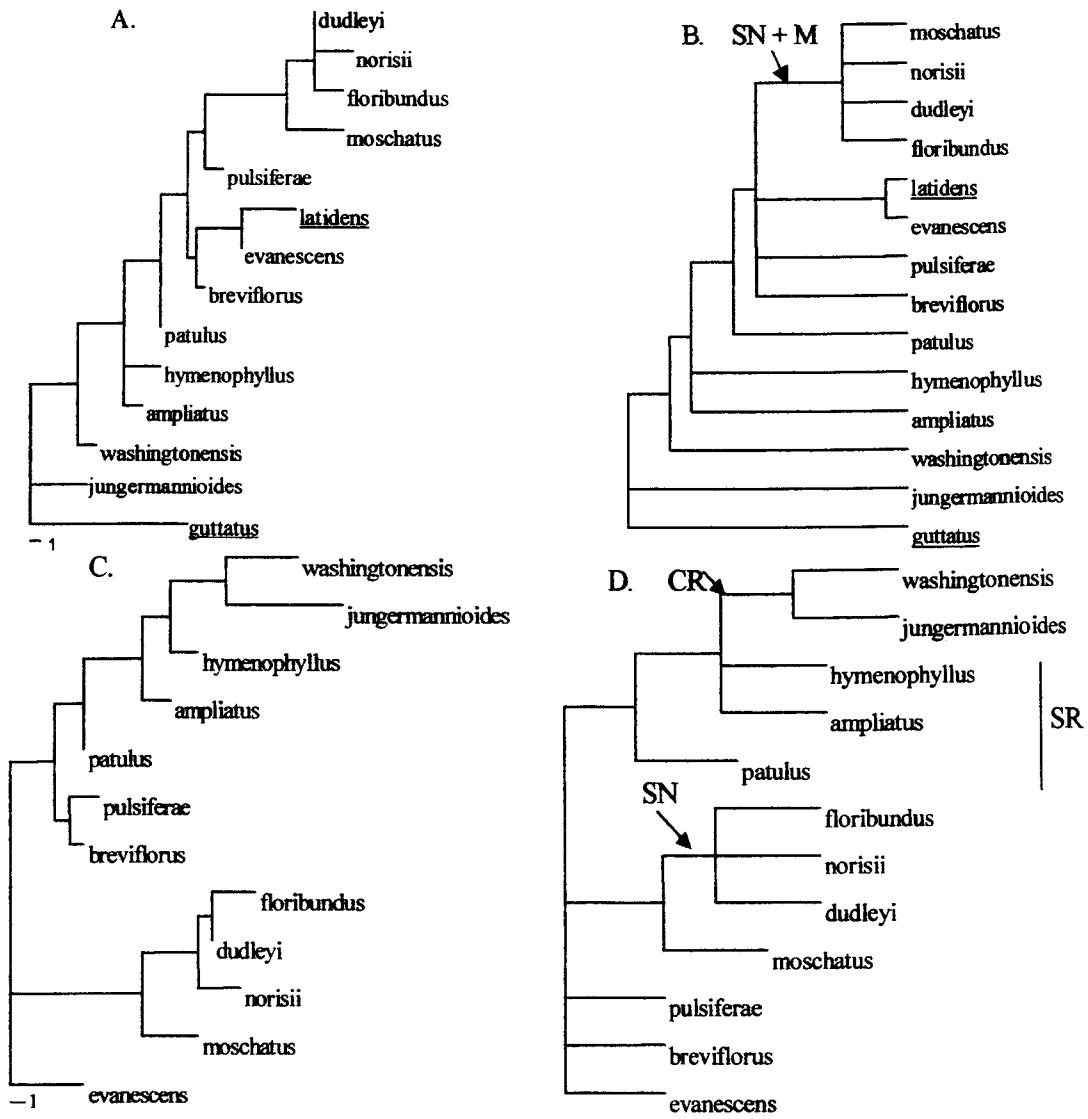


FIGURE 1-5. Full model phylogenetic reconstructions with the two nearest outgroups included and only ingroups. A. and B. = full model phylogenetic reconstructions with the two nearest outgroups. C. and D. = reconstructions with only the ingroup. A.) is one of 57 randomly selected most parsimonious phylogenetic trees showing branch lengths and B.) is the strict consensus tree. Outgroups are underscored. C.) is one of 38 randomly selected most parsimonious trees and D.) is the strict consensus tree. CR = Columbia River clade, SR = Snake River assemblage, SN = Sierra Nevada clade, SN + M = Sierra Nevada clade + *M. moschatus*.

hypotheses testing (Armbruster 1990, 1992, 1993, but see Luckow and Bruneau 1997). However, it is difficult to assess the possibility that morphological and ecological characters included in the analysis may be linked to mating system. Even using "neutral" molecular markers may not be completely independent of the morphological data (Armbruster 1992).

When all outgroups were included, 20 most parsimonious trees were obtained of 62 steps (CI=0.52, RI=0.66). Relationships were very poorly resolved and many of the outgroups could not be rooted outside the ingroup (Fig. 1-6). Most notably, *M. guttatus* (an outgroup belonging to another section) was strongly supported as sister to *M. latidens*, and those two species were sister to *M. evanescens*. The Sierra Nevada clade was moderately well supported (bootstrap value = 53). All other relationships were equivocal.

For the independent data set, as in the full data set, outgroup selection seriously affected tree topology. When only the nearest outgroup (*M. latidens*) was included, eight trees of 37 steps were obtained (CI=0.65, RI=0.72). The results from this data set were similar to those obtained with the full data set (Fig. 1-6 C. and D.). *Mimulus evanescens* was pulled out of the ingroup, the Sierra Nevada clade + *M. moschatus* was well supported; these species plus the Columbia and Snake River clades formed a natural grouping with *M. pulsiferae* and *M. breviflorus* positioned basally.

The tree topology for the unbiased data set when only *M. guttatus* is included as an outgroup was quite different than that for all outgroups included (Fig. 1-7). Only four trees of 43 steps were obtained (CI=0.65, RI=0.69). In this model *M. evanescens* was pulled to the outgroup, the Sierra Nevada clade + *M. moschatus* was a well supported clade, and *M. jungermannioides* was basal to that clade. *Mimulus pulsiferae* and *M. breviflorus* formed a broader clade that was located within a clade including *M. washingtonensis*, *M. ampliatus*, and *M. patulus*. *Mimulus hymenophyllus* occupied a unique basal position (outside of its Snake River affiliation) relative to the rest of the ingroup.

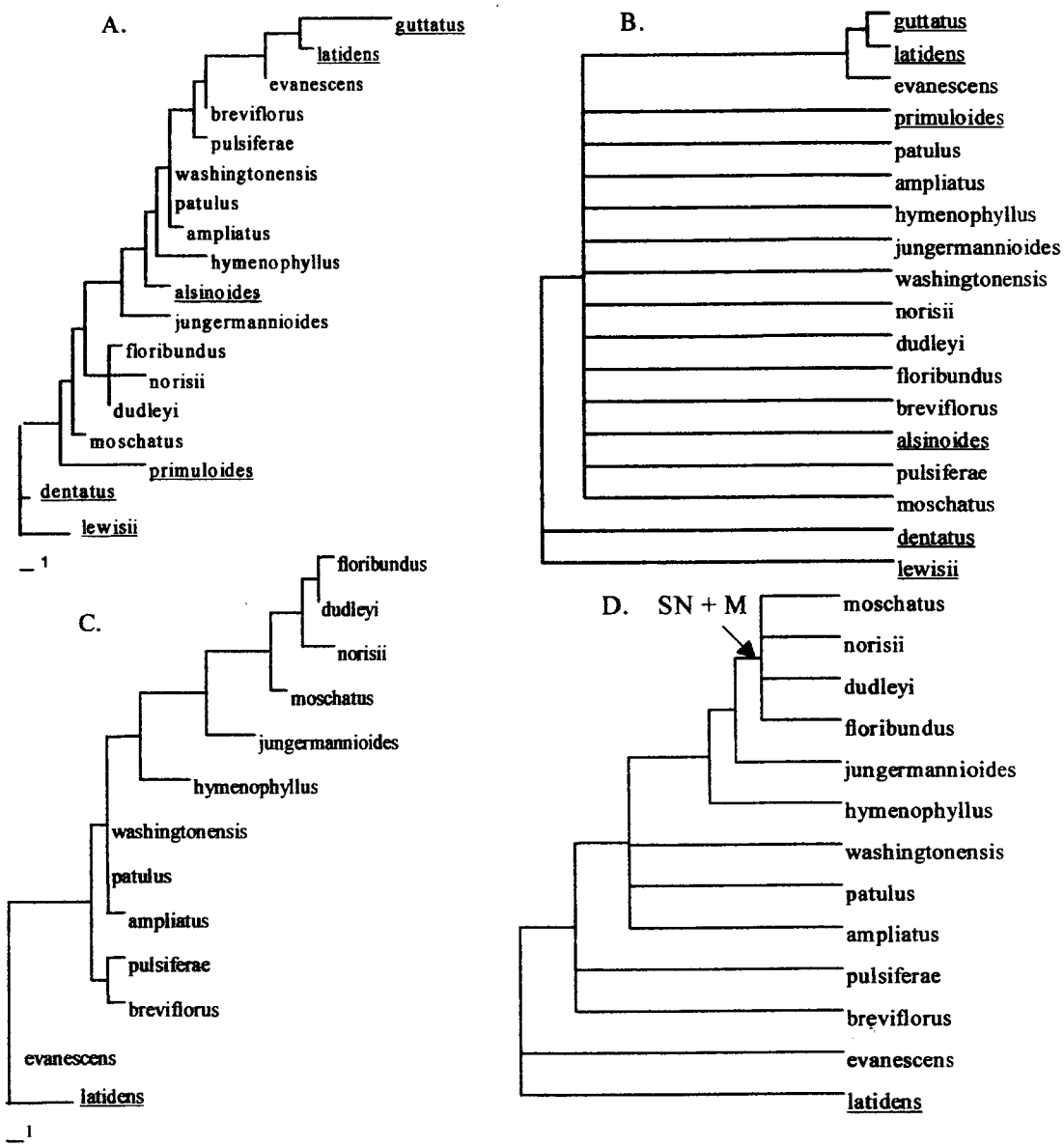


FIGURE 1-6. Phylogenetic reconstructions for the mating system-independent model with all outgroups and two nearest outgroups. A.) is one of 20 randomly selected most parsimonious phylogenetic trees showing branch lengths and B.) is the strict consensus tree with all outgroups included. C.) is one of eight random trees and D.) is the strict consensus tree when only *M. latidens* is the outgroup. SN + M = Sierra Nevada clade + *M. moschatus*.

Last, when both *M. latidens* and *M guttatus* were included as the only two outgroups in the unbiased data set, the tree topology was better resolved than in the full data set (Fig. 1-7 C. and D.). Eight trees of 45 steps were obtained (CI=0.62, RI=0.71). *Mimulus evanescens* was basal in position, followed by a loose assemblage of species, and terminating with the well supported Sierra Nevada clade + *M. moschatus*. Interestingly, the two cliff-dwelling species, *M. jungermannioides* and *M. hymenophyllus*, tended to be allied, while in the full data model they were rarely associated. These species share the vegetative traits of prostrate habit and long-petioled, toothed leaves, traits likely associated with the unique cliff-dwelling ecology.

Composite phylogeny –

A composite approach was taken to synthesize disparate sources of phylogenetic information into a better-resolved tree. Two supertree analyses were performed on data from four molecular sources and the morphological data presented here. One analysis used the strict consensus tree from the full-morphological data set, and the other analysis used only the mating system-independent tree. The impetus behind conducting a supertree analysis with the more restricted data set is to reconstruct evolutionary relationships that are independent of characters that are later critically examined. The synthetic phylogenetic analysis using the full-data morphological strict consensus tree, plus the four molecular trees, obtained six trees of 84 steps (CI=0.68, RI=0.80, Fig. 1-8). In ingroup topology, the six trees differed only in the relationships within the Snake River clade. In half of the trees, *M. ampliatus* was sister to *M. hymenophyllus* + *M. patulus*, and in the other trees *M. patulus* was sister to *M. ampliatus* + *M. hymenophyllus*. Two of the species, *M. patulus* and *M. hymenophyllus*, are sympatric in the Imnaha River drainage, while *M. ampliatus* has only been collected from the opposite side of the Snake River Canyon. Further, the two sympatric species share very similar internal palate pubescence, while *M. ampliatus* has some mixing of the larger clavate external hairs with smaller internal hairs. This suggests that *M. hymenophyllus* and *M. patulus* may be sister species despite lack of phylogenetic resolution.

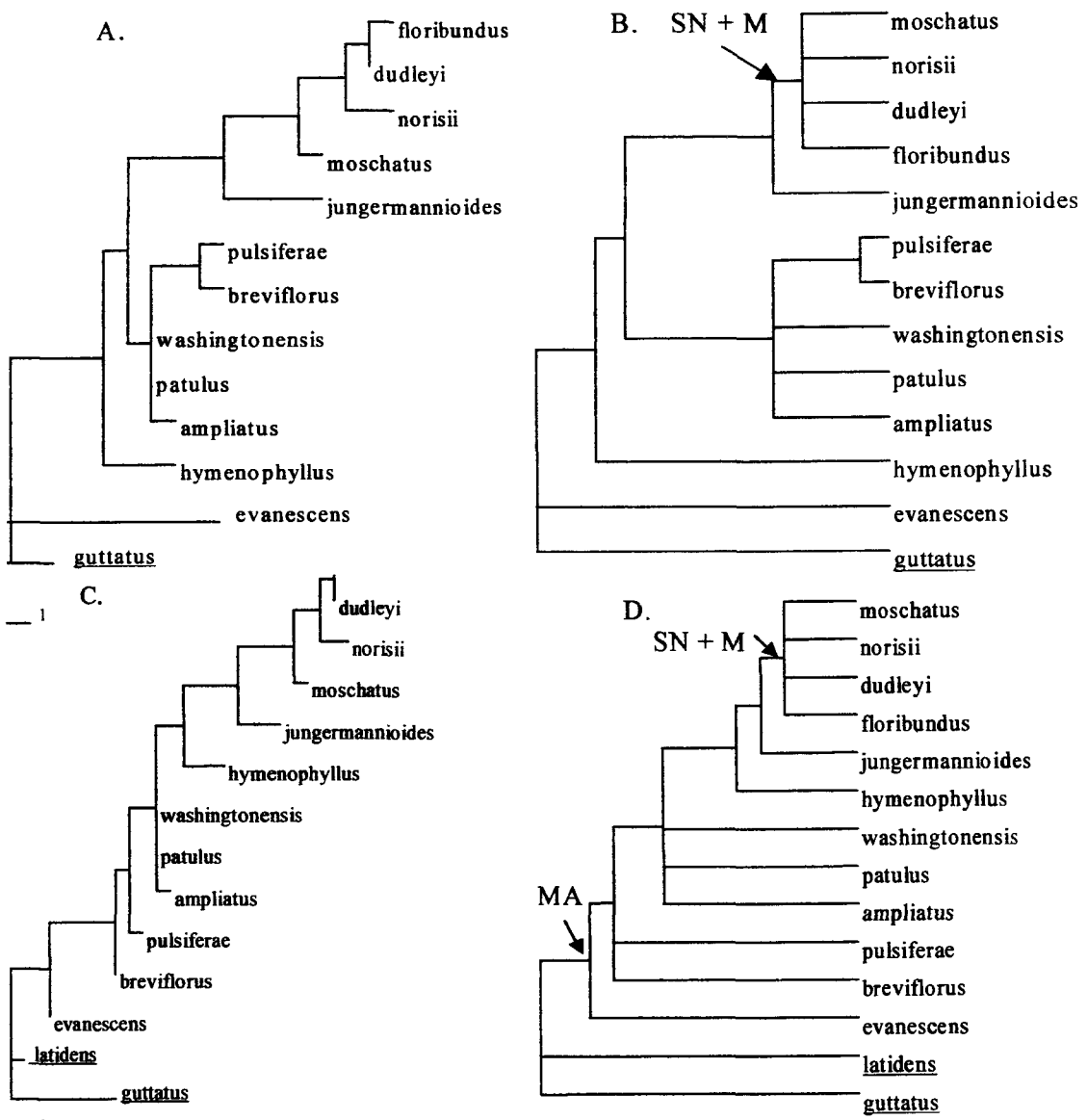


FIGURE 1-7. Phylogenetic reconstructions based on the mating system-independent data with all outgroups and the two nearest outgroups. A.) is one of four randomly selected most parsimonious phylogenetic trees showing branch lengths and B.) is the strict consensus tree with *M. guttatus* as the only outgroup. C.) is one of eight shortest trees and D.) is the strict consensus tree with both *M. latidens* and *M. guttatus* included as outgroups. SN + M = Sierra Nevada clade + *M. moschatus*, MA = *M. moschatus* alliance.

In this analysis the Great Basin clade was well resolved and sister to the Columbia River clade. The Great Basin clade was composed to two minute flowered species, which share reduced corolla features, a swollen calyx in fruit, and gradually tapered leaf-bases. *Mimulus pulsiferae* was sister to the Sierra Nevada clade. The Columbia River clade was composed to two morphologically divergent species: *M. jungermannioides* is a decumbent, cliff-dwelling perennial, while *M. washingtonensis* is an erect annual. These two species do share a number of floral traits, including closed corolla apertures and pubescent styles.

The Great Basin + Columbia River clade was sister to *M. pulsiferae* + Sierra Nevada clade. All these species were sister to *Mimulus moschatus*. This broader clade (including *M. moschatus*) was sister to the Snake River clade. Last, *M. latidens* was resolved as the nearest outgroup to the *M moschatus* alliance.

When the mating system-independent morphological strict consensus tree and the four molecular sources and were combined, six maximally parsimonious trees of 79 steps were obtained (CI=0.70, RI=0.82). Figure 1-8 C.) and D.) display one randomly selected most parsimonious tree and the strict consensus supertree, which is identical to the supertree using the full-data morphological phylogeny. The six most parsimonious trees placed *M. latidens* as sister to the *M. moschatus* alliance, thus providing a functional outgroup (Watrous and Wheeler 1981). The *M. moschatus* alliance was well supported as monophyletic and it possesses a relatively long branch. Within the alliance, a number of clades were present. The Snake River clade (sensu Whittall 1999), consisting of *M. ampliatus*, *M. hymenophyllus*, and *M. patulus*, was well supported and sister to a wider assemblage of ingroup species. Within the wider assemblage, the Great Basin clade (Whittall 1999) of *M. breviflorus* and *M. evanescens*, the Columbia River clade of *M. jungermannioides* and *M washingtonensis*, and the Sierra Nevada clade (Whittall 1999) of *M. dudleyi*, *M. floribundus*, and *M. norisii* were well supported. *Mimulus pulsiferae* was sister to the Sierra Nevada clade, and the Great Basin + Columbia River clade was

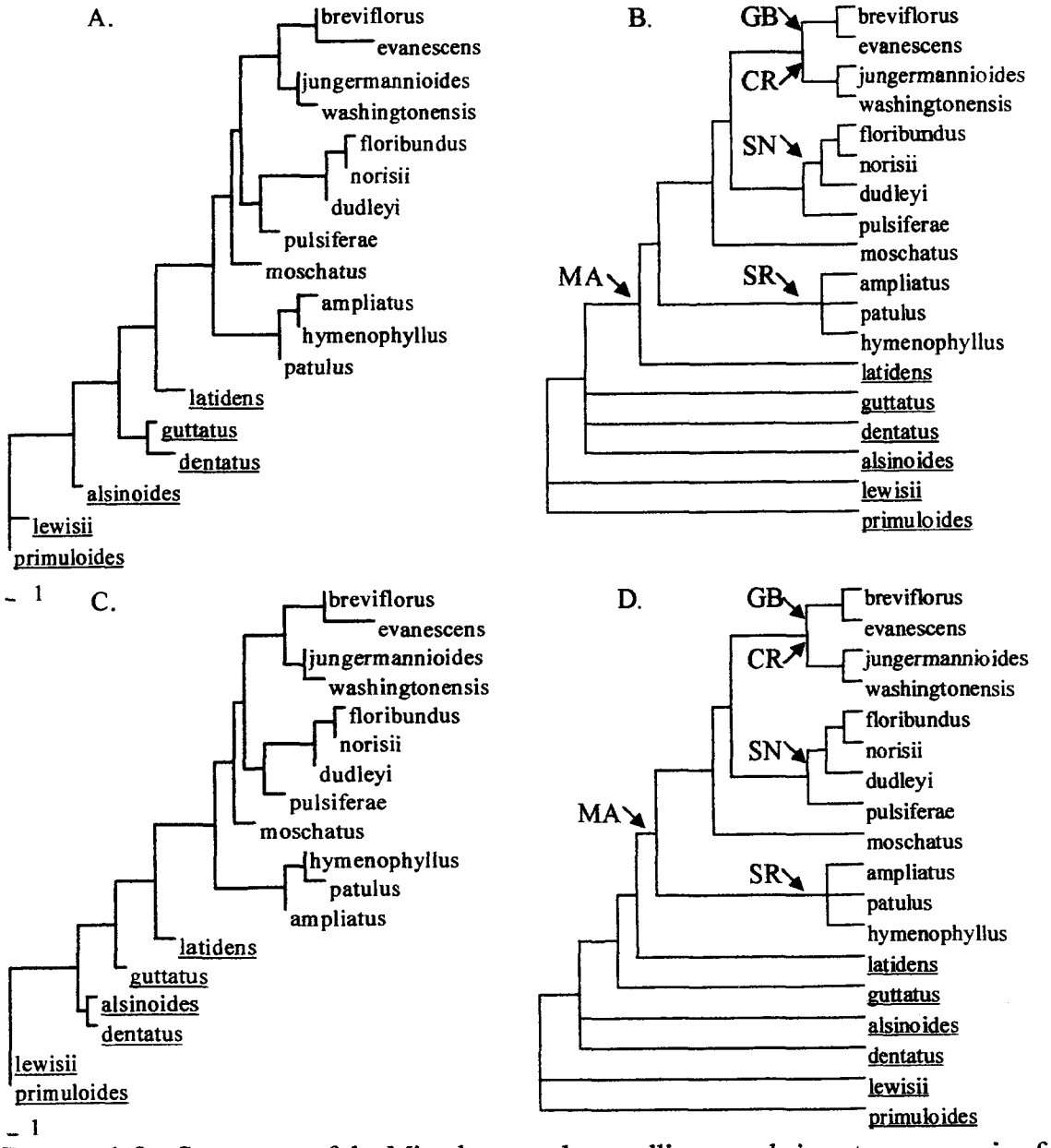
sister to the Sierra Nevada + *M. pulsiferae* clade. *Mimulus moschatus* was sister to the Great Basin + Columbia River + Sierra Nevada + *M. pulsiferae* clade.

The six most parsimonious trees differed in the placement of the outgroup, *M dentatus*, and in the positions of the species within the Snake River clade. In half of the trees *M. hymenophyllus* was sister to *M. patulus*, and in the others, *M. hymenophyllus* was sister to *M. ampliatus*. No reconstructions directly link *M. patulus* with *M. ampliatus*.

No clear morphological synapomorphies define the *M. moschatus* alliance and thus the species assemblage is a "cryptic clade" (see Wojcienchowski et al. 1993). However, when *M. dentatus* is included as a member of the *M. moschatus* alliance the following traits are synapomorphic: stem architecture is progressively bifurcating, stems are thin and not succulent, and seeds are not obviously reticulate. The Sierra Nevada clade (*M. floribundus*, *M. dudleyi*, and *M norisii*) has the synapomorphic traits of light brown, and ovoid to roundly elliptic seeds. The *M. latidens* + *M. evanescens* clade is united with a membranous calyx, sessile upper leaves and a number of additional traits. These traits are also found outside the above-mentioned clades. However, a membranous calyx is found only in one other, distantly related species: *M. guttatus*. While *M. latidens* has been treated as an outgroup (Whittall 1999) to the *M. moschatus* alliance, there has also been the question of its relationship to *M. evanescens* and *M. breviflorus*. *Mimulus evanescens* has been proposed as a hybrid between *M. breviflorus* and *M. latidens* (Meinke 1995, Whittall 1999, Beardsley unpubl.).

Evolution of ecological traits –

The mating system-independent supertree was used to explore character evolution, biogeography, and the evolution of habitat type. When geographic range was mapped onto phylogeny, species within a clade tended to share a geographic region (Fig. 1-9 A.). However, the reconstruction of ancestral states was often equivocal. The nearest outgroup taxa were widespread in western North America, typically non-montane.



FIGURES 1-8. Supertrees of the Mimulus moschatus alliance and six outgroup species for full and independent data sets. A.) is one of six most parsimonious trees using the full data set. B.) is the strict consensus supertree with six outgroup species. C.) is one of 40 most parsimonious trees for the unbiased data set. D.) is the unbiased strict consensus tree, which is identical to the full data tree. See text for a description of data sets and tree reconstruction. GB = Great Basin clade, CR = Columbia River clade, SN = Sierra Nevada clade, SR = Snake River clade, MA = *M. moschatus* alliance.

The more distant outgroups were widespread throughout the Cascades and Sierras and montane West. It seems probable that widespread ancestors gave rise to more restricted progenitors that subsequently have speciated within regions. For example, *M. ampliatus*, *M. patulus*, and *M. hymenophyllus* form a tight clade that is restricted to the Snake River Canyon, while the groups of species that are most closely related to this clade are either widespread montane, or widespread low-elevation species. The picture of species evolution that emerges in this group is one of widespread ancestors, inhabiting novel regions and giving rise to geographically isolated progenitors, which in turn speciate. In all cases it appears that relatively recent migration of species has not occurred.

While rarity (here defined as geographically restricted) is largely a function of various stochastic events, there may be phylogenetic traces that link rare species, and approaching this question from an evolutionary perspective could lead to unexpected results. Rarity appears to have evolved twice in the *M. moschatus* alliance, once in the Snake River clade and again in the broad clade including the Sierra Nevada, the Columbia River, and the Great Basin clades (Fig. 1-9 B.). This pattern of losses and gains was maintained in accelerated and delayed transformations also. In the broader clade, rare lineages became widespread on three occasions: *M. pulsiferae* is a widespread species sister to the Sierra Nevada clade, *M. floribundus* is a widespread species in the Sierra Nevada clade and *M. breviflorus* is a widespread species in the Great Basin clade. Although the sampling within the genus as a whole is limited, it is apparent that geographically widespread species gave rise to narrowly endemic species about as often as narrow endemics gave rise to widespread species.

The evolutionary association with substrate type appears to have moved in the direction of ancestors growing on organic-rich soils toward those growing on organic-poor basalt gravels and rocky outcrops (Fig 1-9 C.). Habitat type has evolved back toward organic-rich soils in *M. moschatus*, and the colonization of rocky outcrops or

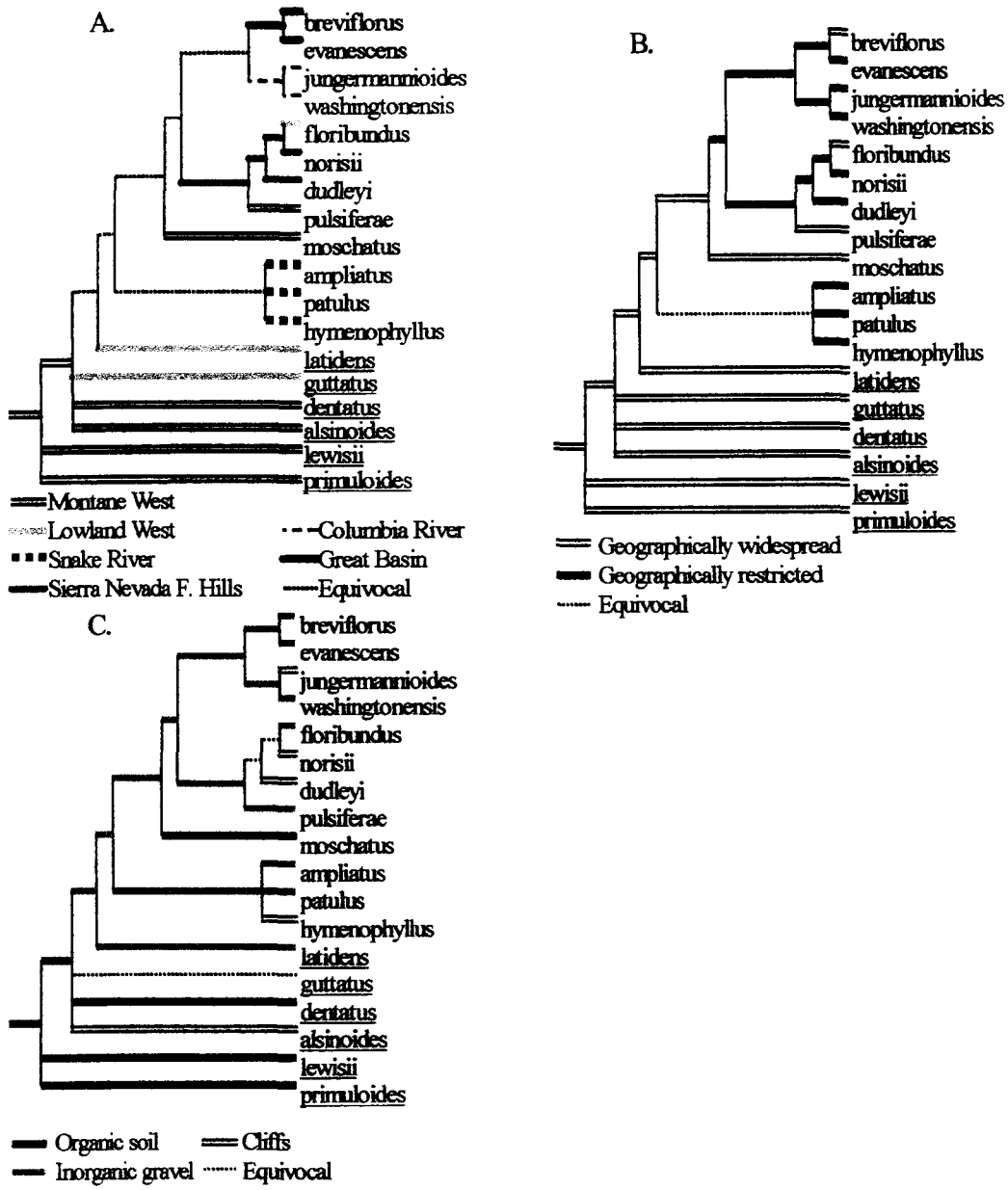


FIGURE 1-9. Strict-consensus supertree with ancestral ecological and biogeographic states mapped. A.) = geographic region. B.) = rarity (i.e., geographically restricted). C.) = primary substrate type. Reconstructions are depicted with no assumptions regarding evolvability of traits; however, analyses were run under both accelerated- and delayed-transformation options. See text for more detailed descriptions.

cliffs has occurred on at least three separate occasions in the ingroup. Four of the eight narrow endemics inhabit rocky outcrops. The widespread montane-western species are found on organic-rich soils with the exception of *M. pulsiferae*, which is found on organic-poor basalt gravels.

The evolution of seed dormancy appears to be quite labile (Fig. 1-10 A.). Within the ingroup all members of the Snake River and Great Basin clades, plus *M. pulsiferae* and *M. washingtonensis* have dormant seeds, while the Sierra Nevada clade, *M. moschatus*, and *M. jungermannioides* do not have dormant seeds. The ancestral states were equivocal for almost all clades. The equivocal cycling procedure in MacClade indicated that there were five transitions, ranging from all gains in seed dormancy or all losses. If seed dormancy is more easily lost than gained, it was likely the ancestral state in the ingroup and dormancy was then lost on three occasions.

Perennial habit was resolved as the ancestral state in the basal outgroups (Fig 1-10 B.). Annual habit was then gained with the nearest outgroup, *M. latidens*, and was subsequently reversed to perenniality two separate occasions: with *M. moschatus* and *M. jungermannioides*. It is interesting that the evolution of perennial habit occurs by innovation of different structures in these two species. *Mimulus moschatus* produces underground rhizomes, like most of the perennial outgroups, while *M. jungermannioides* produces above-ground, negatively phototropic runners that terminate in bulb-like structures (turions) deposited in bedrock cracks. This supports the independence of the reversals to perenniality.

Evolution of pollination systems follows a pattern of large- and small-bee pollinated species towards more exclusively small-bee pollinated, and occasional evolution into almost entirely autogamous species (Fig. 1-11). Shifts in the three pollination systems appear to be moderately labile, with a total of seven transitions, and the consistency index for pollination system was considerably lower than for mating

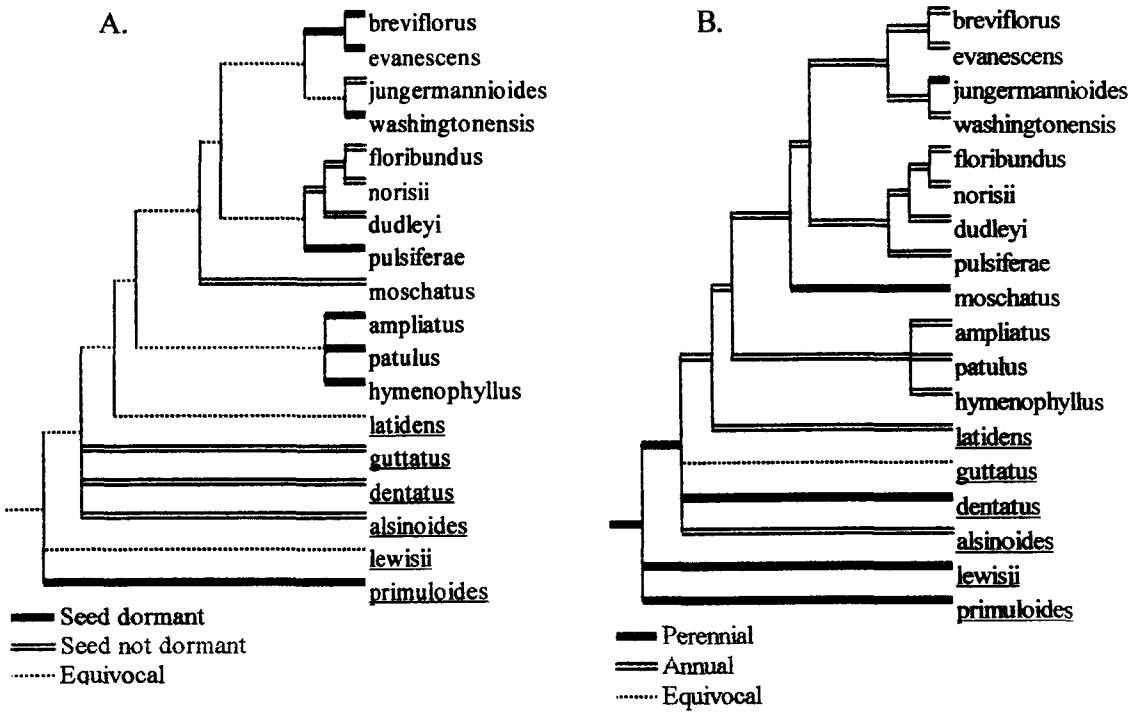


FIGURE 1-10. Strict-consensus supertree with ancestral ecological states mapped. A.) = seed dormancy. B.) = perennial/annual life-history. Reconstructions are depicted with no assumptions regarding evolvability of traits; however, analyses were run under both accelerated- and delayed-transformation options. See text for more detailed descriptions.

system-independent morphological features in general (CI = 0.39 vs. 0.52). The shift towards small-bee and autogamous pollination is concordant with the evolution of more derived species that occupy hot, arid, and variable habitats, with fewer large bees (especially *Bombus* species, which are largely adapted to cool temperate and arctic regions: Heinrich 1979). However, the direction of transitions tends to be consistent. Autogamous species generally occupy terminal branches and do not give rise to additional species. It was likely an autogamous ancestor, however, that gave rise to two species, *M. evanescens* and *M. breviflorus*. The delayed and accelerated transition options did not greatly alter evolutionary patterns. Under the delayed transitions the pollination system at the root of the tree remained equivocal, evolving to small-bee pollinated only around the nearest outgroup to the *M. moschatus* alliance. The branch to the Snake River clade was estimated as small-bee pollinated under this option. The only change in the accelerated transition option was a greater resolution in pollination systems for basal (outgroup) branches. This was likely not particularly informative since species sampling was not thorough in the outgroup.

DISCUSSION

The phylogeny proposed here for the *M. moschatus* alliance represents a synthesis of previous molecular analyses and a morphological, cytological, and ecological analysis, with the various data sources combining into a well resolved topology. Cladistic inference from combined, independent data sets has been stressed as the path to the clearest phylogenetic resolution (Hillis 1987, Vickery and Wullstein 1987, Olmstead 1989). The strongest inferences from each data set may be combined into the phylogenetic reconstruction, achieving the greatest resolution. The weaknesses present in

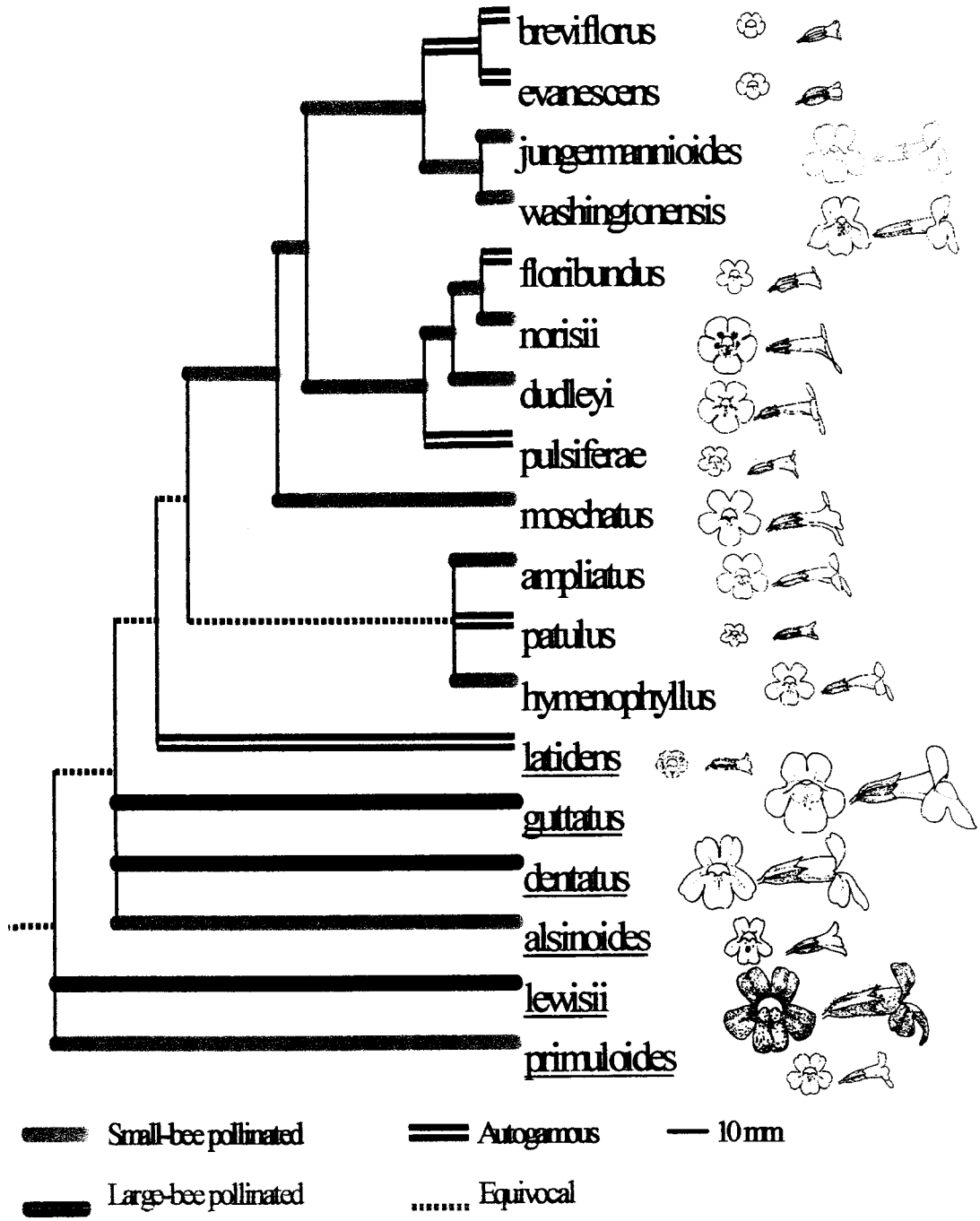


FIGURE 1-11. Strict-consensus supertree showing evolution of pollination system in the *Mimulus moschatus* alliance. Reconstructions are depicted with no assumptions regarding evolvability of traits; however, analyses were run under both accelerated- and delayed-transformation options. See text for more detailed descriptions. Illustrations of corollas, given to the right of species, are to scale of one another.

each method preclude relying too heavily on any one method to obtain the best phylogenetic relationships for the group (Olmstead 1989). This synthetic approach was employed to test taxonomic hypotheses and determine evolutionary patterns in a systematically troubling species alliance.

Morphological and molecular phylogenies –

Monophyly of the *M. moschatus* alliance has been well supported by molecular analyses (Whittall 1999, Beardsley unpubl. data), but was poorly supported by most of the morphological reconstructions in this study. In the morphological full-data model with all six outgroups, the *M. moschatus* alliance was paraphyletic, including three outgroups, *M. latidens*, *M. alsinoides*, and *M. primuloides*. Further, there were no morphological characters found that are synapomorphic for the ingroup, and length of branches separating in- and outgroups is quite small. Previous authors have suggested that these species possess a unique combination of viscid pubescence, reduced calyx teeth, and acrescent fruiting pedicels (Grant 1924, Argue 1986, Meinke 1992); however, individually these traits are all found outside of the ingroup and some traits are not apparent in all of the ingroup. Traits that are found in the majority of ingroup species are pollen type (IIc), anther sac morphology (unequal, widely opening theca), and chromosome number (N = 16). Despite lack of monophyly in the morphological data sets, all four molecular reconstructions, based on nuclear ITS and ETS and two chloroplast genes, displayed very strong support for the monophyly of the ingroup (Whittall 1999, Beardsley unpubl. data). However cryptic, it is probable that the *M. moschatus* alliance is a natural clade.

Overall, tree topology was very sensitive to outgroup identity. Nevertheless, a number of areas of congruence were found within the ingroup when different sets of outgroups were included. The most consistently defined clades follow biogeographic patterns originally defined by Whittall (1999).

Sierra Nevada clade - In all trees *M. floribundus*, *M. dudleyi*, and *M. norisii* (i.e., the Sierra Nevada clade, sensu Whittall 1999) were well resolved as a clade. These species overlap in biogeographic region in the southern Sierra Nevada Foothills. *Mimulus dudleyi* and *M. norisii* are geographically and edaphically restricted, while *M. floribundus* is widespread throughout the west. This clade shares uniquely round to sub-ovoid, light-colored seeds lacking obvious reticulations, pollen grains lacking supratectal ornamentation (which also occurs in the distantly related outgroup species, *M. alsinoides*, *M. lewisii*, and *M. primuloides*; Argue 1980), and white styles with obovate stigmatic lobes. These species also share a unique base-pair deletion in *rpl 16* intron sequence (Whittall 1999). Relationships within the clade are poorly resolved, but based on molecular data Whittall (1999) suggested that *M. norisii* and *M. floribundus* are sister species.

Mimulus moschatus was often sister to the Sierra Nevada clade in morphological reconstructions. These species all share glabrous interior palate ridges, nearly rotate corollas, multi-cellular calyx pubescence, and highly viscid herbage. *Mimulus moschatus* is notably divergent from the Sierra Nevada clade in life history and habitat preference. This species is a perennial, restricted to high-elevation stream sides and wetlands of well developed organic substrates, while the Sierra Nevada clade are annuals restricted to ephemerally moist basaltic or granitic gravels or rock outcrops. *Mimulus moschatus* is not closely associated with the Sierra Nevada clade in molecular reconstructions. With its widespread geography, relatively large (outcrossing) and "unspecialized" flowers (Stebbins 1974), perennial habit, and generalized morphology, this species appears a good candidate for a more basal placement.

In chloroplast-based trees, *M. pulsiferae* was nested in the Sierra Nevada clade. However, *M. pulsiferae* was not rooted in this clade in morphological or nuclear gene-based trees. In the *trn*L phylogeny *M. pulsiferae* formed a clade with the Sierra Nevada clade + *M. moschatus* (Beardsley unpubl.). In the ITS reconstruction *M. pulsiferae* was part of an unresolved trichotomy with the Columbia River clade, Great Basin clade, and *M. moschatus*, while in the *rpl16* reconstruction it was sister to *M. floribundus* (Whittall

1999). The single-celled pubescence, nearly regular corolla, slender calyces in fruit, and bifurcating stem is consistent with the unresolved placement of *M. pulsiferae* in the *M. moschatus* alliance (Whittall 1999). Whittall (1999) and Meinke (1992) suggested that the placement of this species in the Sierra Nevada clade in the *rpl16* intron data set is consistent with the apparent morphological grade in the northern Sierra Nevada towards *M. arenarius*. *Mimulus arenarius* was not sampled in this study, but is likely a member of the Sierra Nevada clade as it shares the distinctive multi-cellular pubescence, a short and wide calyx in fruit, and pollen type IIb (Grant 1924, Argue 1980, 1986, Meinke 1992, Whittall pers. comm.). Both *M. arenarius* and *M. pulsiferae* share a persistent basal rosette, overlap in the northern Sierra Nevada Mountains, and appear to intergrade morphologically in Northern California (Meinke unpublished). It is tempting to suggest that some degree of hybridization has occurred or is occurring, and chloroplast capture explains the discrepancy between the nuclear and chloroplast gene phylogenies.

Columbia River clade - *Mimulus jungermannioides* and *M. washingtonensis* are restricted to ephemerally moist basalt substrates along the Columbia River Plateau in northern Oregon. These taxa were resolved in morphological reconstructions as sister species only with the inclusion of no outgroups, or only the nearest proposed outgroup, *M. latidens*. These two species share pubescent styles (however, *M. jungermannioides* styles are only weakly pubescent) and possess corollas with more tightly closed apertures and lateral petals that angle downward. When *M. guttatus* was included as an outgroup, the monophyly of the Columbia River clade breaks down. This was apparently due to many characters shared between *M. jungermannioides* and *M. guttatus* (e.g., calyx lobes converging in fruit, calyx swollen in fruit, calyx membranous, perennial habit). Undoubtedly, these characters are largely homoplasious, as *M. guttatus* belongs to a different section, and none of the molecular analyses suggest any relationship between the two species. The Columbia River clade is supported in the two strict consensus, nuclear gene-based trees, and they share two parsimony informative ITS region indels (Whittall 1999).

Great Basin clade – Mimulus breviflorus and *M. evanescens* are diminutive Great Basin species that have been suggested as sister species based on similar corolla morphology, fine pubescence, sessile ovaries, papery-urceolate fruiting calyces (Meinke 1995), and similarities in ITS and *rpl16* sequences (Whittall 1999). Despite strong molecular evidence, the morphological reconstructions did not support the presence of the clade. While these species share many morphological features, they lack clear synapomorphies to link them cladistically. Meinke (1995) suggested *M. evanescens* was closely related to *M. latidens*, a relationship supported in the morphological reconstructions. These species share sessile, gradually tapering leaves, swollen, membraneous calyces in fruit, and abruptly tapered capsules. However, all of these traits are also found in other species. The lack of clarity among these three species may be due to hybridization, which was supported by morphological analyses (Meinke 1995) and ambiguities in ITS sequences (Whittall 1999, Beardsley unpubl.). In general hybridization is not widely documented in the genus, however.

Snake River clade - Mimulus ampliatus, *M hymenophyllus*, and *M. patulus*, which are narrow endemics to the Hell's Canyon section of the Snake River, received little support as a natural group in the morphological analysis, but these species were often allied in a paraphyletic grade. This clade was resolved as monophyletic in ITS, ETS, *rpl16*, and *trn*L intron/spacer trees, even sharing a unique *rpl16* indel (Whittall 1999, Beardsley unpubl. data). It is not surprising that morphological analyses failed to unite these species, as they are morphologically very diverse. *Mimulus hymenophyllus* is a cliff-dwelling species with some autapomorphies (very large, elongated, black seeds) and traits unique to the other members of the clade (large, toothed, and thin leaves, and an abruptly tapering capsule in fruit). *Mimulus ampliatus* is unique among *M. moschatus* alliance in having a nearly square stem in cross-section and ovate-shaped stigma lobes. *Mimulus patulus* is a diminutive species without significant synapomorphies. These species all have open corolla apertures, more abruptly tapering leaf-bases, finely

glandular pubescence, and share similar biogeography. It is likely that these species do share an evolutionary history, but that radiation into different habitat types and numerous population bottlenecks has lead to rapid morphological disparity. Within the clade, ITS and *rpl16* data indicate weak support for a sister species relationship between *M. patulus* and *M. hymenophyllus* (Whittall 1999).

There has been some debate considering the relationship of *M. washingtonensis* with two species in the Snake River clade. *Mimulus patulus* has been synonymized as a smaller, more selfing form of *M. washingtonensis* (Hitchcock and Cronquist 1969) and *M. ampliatus* has been affiliated with *M. washingtonensis*, based on a principal components analysis of 26 morphological characters from herbarium specimens (Meinke 1992). None of the four molecular phylogenies link *M. washingtonensis* closely with *M. patulus* or *M. ampliatus* (Whittall 1999, Beardsley unpubl. data). Both *M. patulus* and *M. ampliatus* are clearly more distantly related to *M. washingtonensis*, even though they share some gross morphological similarities. The unquestionable taxonomic existence of *M. ampliatus* and *M. patulus* as unique species is of particular importance, since they are of conservation concern; unlike vertebrates, American state and federal plant-conservation protocols require taxonomic recognition at the variety/subspecies or species level for legal protective status (16 U.S.C. §§ 1531-1544: USFWS 1988).

The position of *M. pulsiferae* was poorly resolved in the morphological phylogenies. In a number of trees *M. pulsiferae* was sister to *M. breviflorus*, united with traits associated with diminutive corollas and similar vegetative characters of three veined, entire leaves with gradually tapered bases. However, in many other topologies it is more deeply rooted and no clear relationships are present.

Relationship among clades – The morphological reconstructions were not very successful in resolving relationships among the species clades within the *M. moschatus* alliance. The Columbia River clade and Snake River clade tended only to be very loosely allied, a relationship that is incongruent with all molecular trees. Molecular reconstructions generated more resolved basal positions and had some regions of congruence. In

particular, the Columbia River and Great Basin clades were often sister. Additionally, molecular trees tended to concur on *M. latidens* as the nearest outgroup, while this species could not be rooted outside the ingroup in morphological reconstructions.

Mating system-independent phylogenies –

A portion of the data used to estimate the phylogenetic history is undoubtedly linked to the characters and evolutionary hypotheses I subsequently test. I therefore, removed data associated with specific hypotheses to avoid the hazards of circular reasoning (Armbruster 1990, 1992, 1993; but see Luckow and Bruneau 1997). This reduced the number of morphological characters by roughly half. Evolutionary relationships were very poorly defined under these circumstances, especially when all outgroups are included. This indicates that a large amount of homoplasy was present in the largely vegetative data. When more restricted sets of outgroups are employed, some of the relationships observed in the molecular and full morphological data reconstructions became evident, e.g., the presence of the Sierra Nevada clade.

Composite (Supertree) phylogeny –

The combined molecular and morphological trees produced just six most parsimonious trees that, among ingroup species, differed only in relationships within the Snake River clade. The high resolution in this analysis was due to congruence among the molecular trees and the largely undefined relationships in the morphological strict consensus tree (i.e., the morphological data contributed little data in the supertree analysis). The strict consensus supertrees from the full-data and mating system-independent data were identical. The composite phylogeny supported all the above mentioned biogeographic clades and additionally linked the Great Basin clade with the Columbia River clade. The Snake River clade was sister to the rest of the *M. moschatus* alliance and *M. latidens* was the nearest outgroup. The pattern of plant cladistic relationships being consistent with biogeography is quite common (e.g., Olmstead 1989, Soltis et al. 1991, Soltis et al. 1992,

Armbruster 1994, Lammers 1996, Ainouche and Bayer 1998, Armbruster and Baldwin 1998).

Character evolution –

I was unable to define any synapomorphies that define the *M. moschatus* alliance, and this group is best described as a morphologically cryptic clade. This term was first applied to a morphologically indistinct clade of North American *Astragalus* (Fabaceae) based on strong chromosomal, nuclear ribosomal and chloroplast DNA (Wojceichowski et al. 1993). The monophyly of the 'Neo-Astragalus' has been confirmed with four additional molecular studies, but corroborating morphological data remains elusive (Pennington and Gemeinholzer 2000). With the increasing abundance of molecular systematic studies, examples of cryptic clades are becoming much more common (e.g., in Fabaceae: Liston 1995, Sanderson and Wojceichowski 1996, Pennington and Gemeinholzer 2000, in Rhamnaceae: Richardson et al. 2000, and in Apiaceae: Downie et al. 2000). Traits that are associated with most ingroup species include a tetraploid chromosome number (N = 16), progressively bifurcated stem architecture, viscid herbage, anthers than open incompletely, and unequal theca.

Evolution of ecological states –

Biogeography, rarity, substrate type, seed dormancy, perennial habit, and pollination system were explored in a phylogenetic context by mapping ancestral conditions onto a composite tree. Phylogenetic and biogeographic patterns support the idea of a Californian origin of the alliance, widespread ancestral migration, and subsequent speciation within regions. Geological activity may have further accelerated cladogenesis in this group and genus, which according to Stebbins (1974), began a period of active evolution during the Tertiary, a period marked by active mountain-building. In this species group, the ancestral condition most likely had a widespread distribution throughout the Sierras, southern Cascades, and montane West. Widespread radiation into the non-montane West appears to follow, with species occupying vernal pools and

disturbed moist areas. All these regions overlap with the California Floristic Province in which the genus is believed to have originated (Grant 1924). Subsequently, speciation is apparent in more restricted and divergent regions. This fits the "species-pump" model of speciation, where a widespread species undergoes range contraction into a series of isolated populations that subsequently diverge into new species (Stebbins 1974). Three species likely evolved in the Snake River Canyon, three in the Sierra Nevada Foothills, two in the Columbia River Plateau, and two in the Great Basin. Within the Sierra Nevada Foothills a more wide-ranging species (*M. floribundus*) evolved and has since spread to a broad area from California to the Rocky Mountains. The two species in the *M. moschatus* alliance that have the most uncertain phylogenetic positions also retain (or re-evolved) the ancestral widespread western montane condition. It is possible that the species alliances with restricted distributions were once much more widespread, and Holocene warming and drying trends caused extensive reduction in ranges of these mesic-dependent species, such that small collections of species were trapped in reduced islands of suitable habitat. This hypothesis would likely result in geographic alliances that are not necessarily linked by phylogeny (e.g., see Trewick and Wallis 2001). Further, it appears that relatively recent migration of species has not occurred, which is not surprising since modern habitat conditions in western North America are characterized by extensive arid regions. The image of species evolution that is resolved in this group of *Mimulus* is one of widespread (Californian) ancestors, inhabiting novel regions largely east of the Cascades and Sierras and giving rise to geographically isolated progenitors, which have in turn speciated.

Within the context of phylogeography an essential question to evolutionary and conservation biology is whether rarity (i.e., with narrow geographic ranges) is a derived state or whether geographic restriction leads to high speciation rates. Widespread species typically have high overall population sizes and are subjected to very diverse selective pressures across their ranges. In many respects, this condition would appear to favor opportunities for speciation, and thus recent derivatives would necessarily be geographically restricted. However, it is generally accepted that some degree of

geographic isolation is also required (Mayr 1942, Dobzhanski 1951). Further, geographically restricted species might be expected to occupy terminal branches because rare species often have more selfing forms of mating system (Karron 1991), which are not expected to maintain sufficient genetic diversity to further speciate (Takebayashi and Morrell 2001). The positive association of autogamy with rarity has been hypothesized to relate to reduced number of potential mates (Baker 1955, Jain 1967), unreliability of pollinators (Tepedino 1979, Karron 1987), and reduced genetic load (Lande and Schemske 1985). In the *M. moschatus* alliance, geographic restriction appears to have arose twice from widespread progenitors, once in the Snake River clade and again in the clade including the Sierra Nevada, the Columbia River, and the Great Basin clades. Rarity does not appear to be an evolutionary dead-end, as further speciation of both rare and widespread species is apparent from rare ancestors. Widespread distributions are subsequently gained three times, in *M. pulsiferae*, in *M. floribundus*, and in *M. breviflorus*. Although the sampling within the genus as a whole is limited and few transitions are present, it is apparent that geographically widespread species in this alliance give rise to geographically restricted species, with roughly equal frequency to restricted species giving rise to widespread species.

I know of no other studies that explicitly investigate the evolutionary history of rarity, and it is thus difficult to evaluate how often rare species give rise to more widespread species. When the condition of geographic restriction is mapped onto the phylogeny for 20 species of *Senecio* sect. *Senecio* (Comes and Abbott 2001) the pattern remains ambiguous. At least four transitions occurred, but it is difficult to estimate the basal conditions. It is possible that all four transitions were gains of narrow endemism from widespread ancestry, but the converse is also possible. The evolution of rarity requires greater study.

A feature of species biology related to geographic restriction is soil or substrate requirements. Many of the geographically restricted species in the *M. moschatus* alliance are found on relatively unique moist basalt or granite outcrops, or seasonally wet cliffs in arid regions. Other species occupy organically rich substrates, typically perennially

moist streamsides, while others are found only on organically poor, ephemerally moist basalt gravels. The direction of the evolution of substrate requirement appears to have been from organic-rich soils toward organic-poor basalt gravels, and rocky-outcrops. Rocky outcrop or cliff-dwelling species have evolved on at least three separate occasions in the ingroup. *Mimulus hymenophyllus* and *M. jungermannioides* separately evolved to cliff-dwelling from organically poor ancestors as did *M dudleyi* and *M. norisii* or their ancestor. Four of the eight geographically restricted species inhabit rocky outcrops. Within the *M. moschatus* alliance, substrate requirement has also evolved from organic-poor substrates back to the ancestral, organic-rich substrates in *M. moschatus*. In general, the widespread montane-western species are found on organic-rich soils with the exception of *M. pulsiferae*, which is typically found on organic-poor basalt gravels.

The role of habitat in explaining phylogeographic patterns has been demonstrated in *Isoetes*, where major clade radiation was associated with invasion of terrestrial and then secondary invasion of aquatic habitats (Taylor and Hickey 1992). Additionally, these serial shifts resulted in a great deal of morphological parallelism and convergence (Taylor and Hickey 1992), a pattern similar to that in *Mimulus*.

Winter seed dormancy is an ecological feature important in high-altitude, moist conditions, which prevents germination in the autumn (Amen 1966, Kaye 1997). Amen (1966) noted that while not all alpine species possess seed dormancy, the most abundant and dominant species are winter dormant. Winter seed dormancy restricts germination timing to moist and warming spring conditions when chances of survival are highest. This trait should be present in species from cold-winter habitats and be evolutionarily labile as species radiate or migrate into and out of such habitats. If this trait is evolutionarily conservative, then it may represent a strong constraint on migration of species into or across new regions. In the *M. moschatus* alliance, the direction of seed-dormancy evolution is largely equivocal. At least five transitions are present and the ancestral conditions are largely not resolved due to the lack of evolutionary signal. The equivocal cycling procedure in MacClade indicated that the possible transitions range from all gains in seed dormancy or all losses. If seed dormancy is more easily lost than

gained, then it was likely the ancestral state in the ingroup and was subsequently lost on three occasions. Regardless, seed dormancy appears only roughly allied with cold-winter conditions. The outgroups tend to be associated with relatively mild winter conditions and these are all non-dormant as expected. (However, there are a few isolated populations of *M. guttatus* with winter dormant seeds: Meinke unpubl. data.) Similarly, the "winter-warm" Sierra Nevada clade is characterized by non-dormant seeds, where fall or winter germination does not result in high pre-reproductive mortality. These species are facultatively multivoltine, with germination and growth only ceasing at the onset of summer drought. However, other species in the ingroup that are found in winter-cold conditions lack seed dormancy. The two winter-cold species lacking dormancy, *M. moschatus* and *M. jungermannioides*, are also the only two perennial species in the ingroup. It is evident that seed germination under inappropriate conditions for an annual would result in complete loss of life-time fitness; however, a perennial would have opportunities for multiple reproductive bouts and if year to year conditions are highly variable, it may be selectively advantageous to have opportunities for more than one generation per year. Overall, phylogenetic history is a poor predictor of the presence or absence of seed dormancy, with many clades possessing members with both character states. This supports the notion that this trait is evolutionarily quite flexible and does not represent a serious constraint to the ability to occupy new habitats with different seasonal conditions.

Perennial habit is generally viewed as a primitive state, repeatedly giving rise to annuals (Carlquist 1962, Stebbins 1974). This pattern of transition in life history is based on fossil evidence, high frequency of woodiness in more basal angiosperm families, increasing aridity in the Miocene and Pliocene that favored a short-lived habit, and theory relating to an association of outcrossing with more long-lived species (Takhtajan 1969, Raven and Axelrod 1974, Stebbins 1974, Barrett, Harder, and Worley 1997). However, more detailed phylogenetic studies have shown that life history evolution can be labile, perennials are not necessarily restricted to basal positions, and annuals have repeatedly given rise to perennials. In *Lupinus* (Fabaceae), there are a number of annual/perennial

species pair that have the same ITS sequence, indicating that annual and perennial habits have evolved independently many times (Ainouche and Bayer 1999). In a reconstruction of the Polemoniaceae, the basal clade is composed of perennials (tropical trees and shrubs); the annual habit evolved at least seven times in the temperate clade, and is a basal character for a large clade (Barrett, Harder, and Worley 1997). Reversal to the perennial habit occurred at least three times in the Polemoniaceae (Barrett, Harder, and Worley 1997). When life-history traits are mapped onto Comes and Abbott's (2001) molecular phylogeny of *Senecio* sect. *Senecio* (Asteraceae), annual habit appears to be the basal condition of the ingroup, with at least three transitions to the perennial habit.

In this alliance of *Mimulus*, the perennial habit condition was resolved as the ancestral state. Annual habit was then gained with the nearest outgroup, *M. latidens*, and reversal to perenniality occurred twice (separately in *M. moschatus* and *M. jungermannioides*). This pattern supports the accepted notion of annual habit as derived, but also indicates that annuals gave rise to additional annuals with high frequency and are capable of evolution back to perennials. The annual species are generally associated with ephemerally moist environments (i.e., vernal pools or spring run-off). Within the ingroup, the two perennial species evolved this life-history by the innovation of different morphologies. *Mimulus moschatus* produces underground rhizomes, like most of the perennial outgroups, while *M. jungermannioides* produces above-ground, negatively phototropic runners that terminate in bulb-like structures (turions) deposited in bedrock cracks. This suggests that in annuals, the ancestral, perenniating rhizome genes may have been lost and not simply silenced, since the subsequent evolution of the perennial habit occurred via innovation of novel structures.

In all of these studies, annuals are not restricted to terminal branches and evolutionary change in life history appears relatively labile. Finally, annuals are clearly associated with more arid and variable conditions, as suggested by Baker (1955).

Pollination systems have been characterized as highly evolvable (Grant and Grant 1965), and more recent studies that employ more rigorous phylogenetic methods have come to similar conclusions (Armbruster 1992, Bruneau 1997, Johnson et al. 1998,

Barrett et al. 1997, Armbruster and Baldwin 1998; but see Luckow and Hopkins 1995 for a more evolutionary conservative example). However, most of these studies involve tropical or subtropical groups, and patterns for temperate species are still unsettled. For example, Macior (1982) described all species of the large temperate-arctic genus, *Pedicularis* (Scrophulariaceae), as employing the same group of bumblebee pollinators. Despite relative consistency in identity of pollinators, *Pedicularis* has evolved a diversity of trait-suites among species that constrain pollinator behavior into multiple stereotypic patterns. There are suggestions however, that at least one species of North American *Pedicularis* is adapted to hummingbird pollination, and Asiatic species have evolved very long corolla tubes, similar to those in genera that are pollinated by lepidoptera or long-tongued dipterans (Stebbins 1974, and references therein). Additionally, the evolution of cross and self-pollination systems is of long-standing interest, where the pattern from cross to self-pollination is commonly assumed to be unidirectional (Stebbins 1957, and see review by Takebayashi and Morrell 2001). There are no clear examples of selfing lineages giving rise to outcrossing lineages, but only a handful of phylogenetic studies exist (Takebayashi and Morrell 2001).

In the *M. moschatus* alliance the pattern of pollination-system evolution roughly follows a pattern of large- and small-bee pollinated species towards more exclusively small-bee pollinated, and occasional evolution into almost entirely autogamous species. Shifts in the three pollination systems are quite labile, with a total of seven transitions. However, the direction of transitions tends to be consistent. Autogamous species have not given rise to additional outcrossing species and generally occupy terminal branches. However, an autogamous ancestor likely gave rise to one or two species, *M. evanescens* and/or *M. breviflorus*. Relative to the morphological characters used in the phylogeny, pollination system was considerably more labile. Pollination consistency index was substantially lower than for mating system-independent morphological features in general (CI = 0.39 vs. 0.52), indicating a higher level of homoplasy in pollination system.

Pollination systems are clearly not completely discontinuous, and thus this type of analysis obviously makes broad generalizations. Species characterized as "autogamous"

in this analysis include both species with minute-nearly cleistogamous-flowers and those with moderately small-flowers, which receive occasional insect visitation. The "large-bee" pollinated species do not have any mechanisms for limiting visitation by small-bees and in some locations small-bees could be more numerous visitors. However, it is unknown how effective pollination by small-bees is in large-flowered *Mimulus*. Further, in many species there is considerable variation in corolla size and thus likely pollination system. More precise mating system evolutionary studies based on actual outcrossing estimates are currently underway (Carlson unpubl. manuscript). Regardless of these limitations, the general pattern of evolution from large- to small-bee pollination, and occasional evolution to terminal, autogamous species, appears evident.

Summary –

The approach of this study has been to integrate phylogenetic information from multiple sources to test long-standing ecological and evolutionary questions. Evidence from molecular sources and morphological, cytological, and ecological data presented here give support to the existence of a monophyletic *M. moschatus* alliance. Within this alliance, four major clades are identified that have biogeographic associations. Further, this phylogenetic approach supports taxonomic recognition of *M. ampliatus* and *M. patulus*, two rare species, previously synonomized. Morphological reconstructions were not well resolved, due to a large amount of homoplasy in the morphological data, which indicates that many traits, including corolla size, perennial habit, and seed dormancy, are highly evolvable in this group. Ancestral-state reconstruction suggests that widespread western North American species gave rise to multiple geographically restricted clades. Geographically restricted species in turn underwent limited speciation within their region, and no evidence of modern migration is present, although one derivative species has likely undergone major range expansion. It appears that perennials on organically-rich substrates gave rise to annuals associated with organically-poor gravels, and subsequently the perennial life-history was re-gained on two separate occasions. The direction of evolution of seed dormancy is equivocal, but it is a highly evolvable trait with at least

five transitions occurring. Pollination systems appear to shift from large- to small-bee pollinated, and occasional subsequent transitions to autogamous pollination systems. No unambiguous shifts from large-bee pollination to autogamy were detected; instead autogamy has generally evolved from small-bee pollinated, small-flowered lineages. No cases of autogamy giving rise to more outcrossing pollination systems are found, but one case of an autogamous ancestor giving rise to two autogamous species is indicated.

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CHAPTER 2. COMPARATIVE ANALYSIS OF MATING SYSTEM TRAITS IN THE *MIMULUS MOSCHATUS* ALLIANCE

ABSTRACT

Reproductive morphology is often used to infer mating system, or the degree of inbreeding in populations or species. Many studies have linked anther-stigma separation, flower size, and pollen-ovule ratios (P/O's) to outcrossing rates, but rarely have these incorporated the effect of phylogenetic history. Using a phylogenetic approach, I evaluated various traits traditionally linked with mating system to an estimated population-level outcrossing rate derived from allozyme data, in two populations each of seven species in the *Mimulus moschatus* alliance. Additionally, I incorporated and analyzed published data from the *M. guttatus* species complex. These self-compatible species are characterized by a large variation in reproductive traits with some members possessing minute, nearly cleistogamous flowers, and others with relatively large and herkogamous flowers. Estimated population outcrossing rates (t) ranged from completely inbreeding (0.0) to largely outcrossing (0.69), autonomous seed sets ranged from 0.005 to 0.92 seeds/ovule, anther-stigma separations ranged from –0.17 to 1.12 mm, and P/O's range from 5.9 to 28.1. Within the *M. moschatus* alliance, *M. patulus* and *M. floribundus* are obligately autogamous species; *M. hymenophyllus*, *M. ampliatus*, and *M. jungermannioides* are facultatively autogamous; and *M. dudleyi* and *M. washingtonensis* are facultatively xenogamous. Species with larger corolla size displayed higher outcrossing rates, P/O's, and male relative to female resource investment. Closure time of touch-sensitive stigmas was not related to outcrossing rates or any other morphological mating system proxy. Overall, variation in morphological and allozyme outcrossing rate estimates was small among populations, but relatively large among species. Some of the outcrossing rate and mating system trait variation was explained by the phylogenetic history of clades, and thus the strength of a few correlations were in fact weak. After

controlling for phylogeny, outcrossing rates were significantly related to P/O, pollen number, corolla height, and anther-stigma separation. Scaling effects of corolla size were substantial for pollen number and anther-stigma separation. There was a strong, positive correlation between P/O and pollen volume, however pollen number per flower was not related to pollen volume. An index of male relative to female resource investment was significantly related to outcrossing rates only in the phylogenetically 'naïve' analysis.

INTRODUCTION

The evolution of self-fertilization from outcrossing lineages is believed to be one of the most common evolutionary transitions in flowering plants (Stebbins 1950, Grant 1981, Barrett, Harder, and Worley 1996, Takebayashi and Morrell 2001). Floral morphology is inferred to evolve in concert with the degree of selfing. A self-fertilizing mating system can be achieved in a number of ways: loss of physiological self-incompatibility (e.g., Goodwillie 1999), increasing the proximity or decreasing temporal separation of male and female function, and alterations in floral morphology that eliminate visitation by pollinators (Lloyd and Schoen 1992). Anther-stigma separation in space (herkogamy) is perhaps the mostly widely cited example of a floral trait negatively correlated with selfing-rates (Rick et al. 1978, Thomson and Stratton 1985, Motten and Antonovics 1992, Karron et al. 1997, Takebayashi and Delph 2000). Temporal anther-stigma separation (dichogamy) is also negatively correlated with degree of selfing (Schoen 1982a, Ganders et al. 1985, Pellmyr 1987, Fort et al. 1991, Bertin 1993, Maki et al. 1999), although some researchers have shown that dichogamy is not associated with decreasing selfing levels (e.g., Griffin, Mavraganis, and Eckert 2000). Additionally, selection for self-fertilization is accompanied by relaxed selection on pollinator attraction, thus a reduction in corolla size (Ornduff 1969, Stebbins 1974, Wyatt 1983), nectar secretion rates (Wyatt 1984a), and pollen production (Cruden 1977). As such, selfing can be viewed in terms of a shift in sex allocation away from male investment.

The relative number of pollen grains to ovules (P/O ratio) has been used as a crude proxy for mating system (Cruden 1977). The correlation between mating system and P/O has normally been evaluated in relation to pollination efficiency or sex allocation theories (Gallardo et al. 1994). Cruden (1977) argued that lower numbers of pollen grains are sufficient to result in maximum seed set in selfing species that are not exporting pollen, or producing pollen as a reward for insect consumption. For outcrossing species, greater investment in pollen numbers is necessary to compensate for the high attrition rate of exported pollen, i.e., low efficiency. Charnov (1982) correctly noted that hermaphroditic plants receive fitness through both female (ovules) and male (pollen) functions and used sex allocation theory to predict P/O's, and male/female investment, more generally, in relation to mating system. In this case, a species' fitness optimum depends on the relationship between allocation to a particular gamete type and the sex-specific gain curves, which describe how relative male fitness and female fitness increase with investment in male and female function respectively (Charnov 1982). The shapes of gain curves are dependent on many functions, such as degree of sibling competition, inbreeding depression, and resource cost of maternal care. The sex allocation framework also hypothesizes greater P/O's in outcrossing species, due in part, to male-male competition. However, because outcrossers should invest more in male function than selfers, an increased trade-off between pollen size and P/O should exist for outcrossers (Charnov 1982). This should result in an overall decrease in the pollen size-number relationship with the evolution of selfing. Last, the 'genetic value' of pollen is reduced in a selfing individual, due to decreased probability of an outcrossing event (Charlesworth and Charlesworth 1981).

Additional theories suggest that outcrossers should produce larger pollen grains than selfers. Large pollen has been associated with long styles (e.g., Williams and Rouse 1990 and Kirk 1992, cited in Barrett, Harder, and Worley 1997), likely due to greater resource requirements for long pollen tube growth, although, scaling effects may also help explain this pattern. Autogamous species generally have smaller flowers, thus shorter styles, and therefore should produce smaller pollen. Additionally, large pollen

may be associated with outcrossers since they are subject to greater male-male competition and female choice during pollen-tube germination and growth, and larger pollen would presumably confer a competitive advantage (Barrett, Harder, and Worley 1997).

Cruden (1977) developed a rough mating system index, based on floral structure, and reported mean pollen-ovule ratios for five mating systems: P/O = 4.7 for cleistogamy, P/O = 27.7 for obligate autogamy, P/O = 168.5 for facultative autogamy, P/O = 796.6 for facultative xenogamy, and P/O = 5898.2 for xenogamy. However, the utility of this classification for comparing different species groups is questionable, since many factors affect differences in P/O's, and species with different evolutionary histories may have disparate P/O's despite similar mating system (Preston 1986). P/O's are strongly related to the primary pollen vector and how pollen is packaged (e.g., in pollinia, or bound in tetrads or with viscin threads), factors that may have a strong phylogenetic signal (Preston 1986). Within closely related groups, however, P/O's may be a highly accurate and economical estimate of mating system.

While P/O's have been correlated to mating system as measured by morphological features (e.g., Cruden 1977), rarely has this measure been directly compared with outcrossing rates (Preston 1986, although see Schoen 1982b, Ritland and Ritland 1989, Parker et al. 1995). Further, while outcrossing rates are often variable among populations, variation in P/O's is not well studied (however, see Schoen 1982b, Wyatt 1984b).

Autonomous seed set may also be an appropriate proxy of mating system. Species with low outcrossing rates are generally highly self-fertile and often have early selfing mechanisms (e.g., *Collinsia*: Armbruster et al. 2002). However, if inter-plant pollen movement is high prior to a bisexual phase or activation of a delayed selfing mechanism, outcrossing rates and levels of 'unvisited,' autogamous seed set may be de-coupled. The connection between these two features is not well studied. Members in the genus *Mimulus* are known to have a delayed selfing mechanism, autonomously setting seed through a process of corolla abscission (Dole 1990, Meinke 1992).

The genus *Mimulus* is one of only a few genera that have thigmotropic stigmas. Stigmatic lobes close after touch, usually within ten seconds, and generally remain closed if pollen is deposited. This feature has been hypothesized to be an outcrossing mechanism, reducing stigmatic surface area available when a pollinator, covered with self-pollen, exits the basal portion of the corolla tube (Newcombe 1922, 1924, Lloyd and Yates 1982, Ritland and Ritland 1989, Meinke 1992, Fetscher and Kohn 1999). In the *Mimulus guttatus* complex, Ritland and Ritland (1989) found that stigma closure time is shorter in taxa that have higher male biomass allocation, but did not detect a significant correlation with P/O or outcrossing rates.

Here I describe variation in outcrossing rates, reproductive morphology, P/O's, male/female resource investment, autonomous seed set, and stigmatic closure time in two populations each of seven related *Mimulus* species in the *M. moschatus* alliance. Additionally, I include Ritland and Ritland's (1989) data from eight species in the *Simiolus* section of *Mimulus*, which is sister to the *M. moschatus* alliance (Beardsley, unpubl. data), in a more comprehensive, phylogenetically corrected manner. Specifically, I test if P/O's are a reliable estimate of mating system within these species groups. Second, is anther-stigma separation positively correlated with outcrossing rates? Third, is autonomous seed set negatively correlated with outcrossing rates? Is outcrossing accompanied with increased allocation to male function (i.e., corolla size, pollen number and volume, and male/female resource investment)?

Additionally, I test the hypothesis that rapid stigma closure is an adaptation to outcrossing, and therefore stigma closure time should be negatively correlated with outcrossing rates. Further, variation in outcrossing rates and mating system traits are hypothesized to vary continuously (see Vogler and Kalisz 2001, Armbruster et al. 2002) rather than discontinuously, and falling into discrete inbreeding and outbreeding 'syndromes' as suggested by Lande and Schemske (1985,) and Schemske and Lande (1985). I evaluate the mating system and associated character variation in a phylogenetic context, to determine the effect of evolutionary history. Incorporating phylogenetic structure in comparative studies is important in reducing the statistical hazard of non-

independence among data derived from related taxa (Felsenstein 1985). Further, trait variation and covariation patterns viewed in an evolutionary perspective can indicate traits and relationships that are more or less evolutionarily labile, and the order of character state changes (Donoghue 1989, Armbruster 1993, Weller and Sakai 1999).

METHODS

Seeds were collected from two populations of each of seven species. Population locations are given in Table 2-1, and illustrations of the species, with special reference to floral morphology, are provided in Appendix 1-1. The species' ranges are given in Fig. 1-1. Populations of the same species were located at least 2 km apart, except for *M. hymenophyllus*, which is restricted to a single creek drainage in northeastern Oregon. Most of the species in this study do not differ dramatically in floral morphology across their respective ranges. *Mimulus floribundus* has been reported to range widely in corolla sizes (0.5 - 1.5 cm in length; Thomson 1993); however some of the larger reported corolla sizes are due to synonymy with a clearly different species, *M. dudleyi*, also included in this study, (Whittall 1999, Carlson unpubl. manuscript). Corolla sizes of the two populations of *M. floribundus* in this study are near the smaller end of the spectrum.

Seeds were germinated and grown in an Oregon State University greenhouse, kept between 22° C and 17° C with a 16 h daylength. Twenty to sixty individuals per population were grown separately in 730 cm^3 pots with a standard potting mix. At full flowering, roughly five weeks after germination for all populations, a single young (i.e., less than two days old) flower per individual was randomly selected and measured with 0.01 mm digital calipers. Between 15 and 23 flowers per population were measured. Measurements of intact flowers included corolla height, width, length, tube depth, and aperture height and width. Only corolla height and length are reported in Results; additional data are presented in Appendix 2-1. Flowers were then dissected and stigma placement, anther placement, and anther-stigma separation were measured under 16 – 20 X magnification. Figure 2-1 shows the measurements.

TABLE 2-1. Population locations of the seven species in the *M. moschatus* alliance, used in this study.

Species, Population	Locations
M. ampliatus – 1	Road cut above Lake Waha, Nez Perce Co., Idaho
-2	8.5 mi. NW of Kamiah, Lewis Co., Idaho
M. dudleyi – 1	Old Stage Rd., 15 mi. S of Porterville, Tulare Co., CA.
- 2	Reservation Rd., 6 mi. E of Porterville, Tulare Co., CA.
M. floribundus – 1	Bake Oven Creek, Maupin, Wasco Co., OR
- 2	Sherar's Bridge, 7 mi. N of Maupin, Wasco Co., OR
M. hymenophyllus – 1	1 mi. up Hays Gulch from Horse Crk., Wallowa Co., OR
- 2	2 mi. up Hays Gulch from Horse Crk., Wallowa Co., OR
M. jungermannioides – 1	2 mi. E of John Day R., along Columbia R., Gilliam Co., OR
- 2	John Day Dam, along Columbia R., Sherman Co., OR
M. patulus – 1	1.5 mi. N of Horse/Imnaha R. confluence, Wallowa Co., OR
- 2	Cow Creek, Imnaha R. Wallowa Co., OR
M. washingtonensis – 1	10.5 mi. S of Dayville, S Fork John Day R., Grant Co., OR
- 2	4.3 mi. W of Spray, John Day R., Wheeler Co., OR

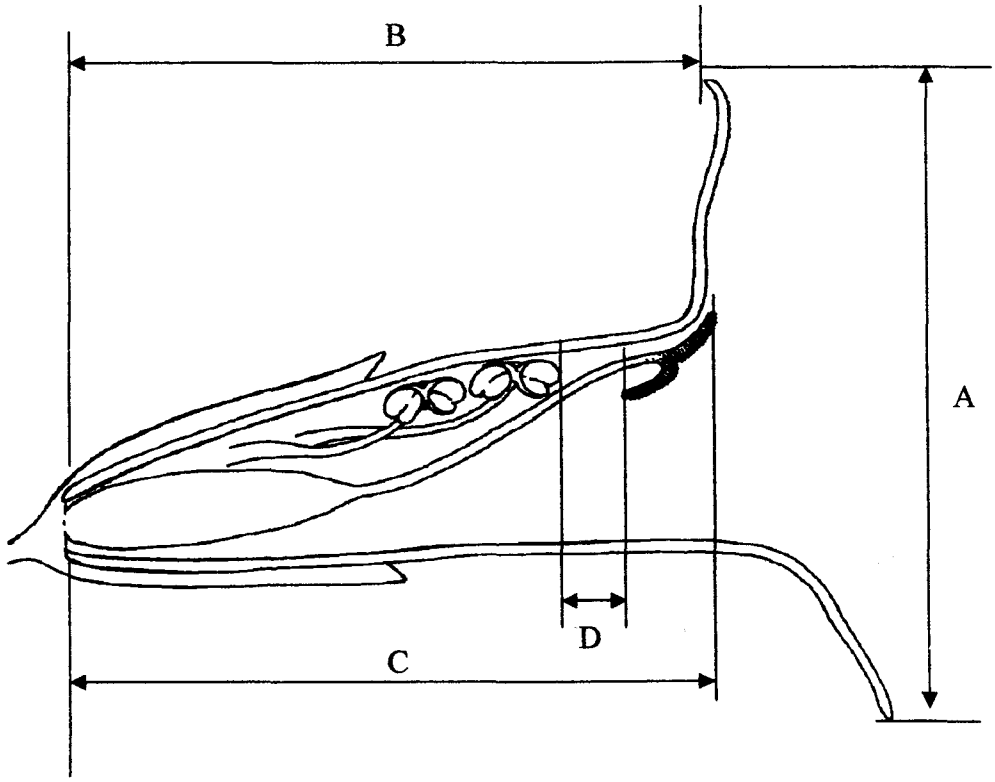

FIGURE 2-1. Measurements made on flowers in the *M. moschatus* alliance. (A) = corolla height, (B) = corolla length, (C) = pistil length , (D) = anther-stigma distance.

Pollen and ovule counts were made on a young, unopened flower from the same branch in which the corolla measurements were taken. Pollen counts were generated by macerating one distal anther in a 40 µl solution of equal parts glycerine to water, and the number of grains on a hemocytometer grid counted and multiplied by the total volume of liquid and by the number of total anthers (four). Pollen number was also estimated from a sample of proximal anthers and was found to not differ from distal anther counts. For each flower, five replicates of pollen counts were made and the mean taken. Pollen grains per flower were counted for 12 to 18 individuals per population. Ovule number was obtained by dissecting the ovary, counting ovules in both locules, or by counting ovules in one of the symmetrical locules and multiplying by two. Counts were made for between 17 and 26 individuals per population. The ratio of pollen grains to ovules (P/O) was made for each flower individually.

Pollen and seed dimensions have been published for six of seven species in the *M. moschatus* alliance (Argue 1986), and these data were used to calculate an index of gamete size and sex-relative investment. Pollen dimensions of *M. ampliatus* (omitted from Argue 1986) were made by macerating anthers from five individuals, mixing them on clear adhesive tape, and measuring polar and equatorial diameters of 15 haphazardly selected grains with an ocular micrometer under 504 magnification. Seed length, width, and thickness were collected from 15 seeds with digital calipers under a 20 magnification dissecting scope. Pollen and seed data were collected from four additional species (ten samples per species), and mean values were found to agree closely with values in Argue (1986), suggesting the *M. ampliatus* data are compatible with the previously published data. Index of pollen volume was approximated by the cubic-root of the formula for an ellipsoid. Index of seed size was taken from Argue (1986) as the cubic-root of length × width × thickness.

To approximate male relative to female resource investment, an index was calculated that incorporates energetic costs: 1000 × (pollen number × pollen volume)/(ovule number × seed volume). The calculation of female gamete investment (denominator) uses seed volume, which also includes male investment, however the

proportion of energetic investment lies primarily on the female parent (see Ashman 1994).

Autonomous seed set was estimated by randomly selecting a single young fruit (5-7 days after corolla abscission) in a pollinator-free greenhouse for 13-14 individuals per population. The capsule was dissected and number of fertilized and unfertilized ovules were counted. Fertilized ovules are easily identified at this stage, as they are much larger, plump, and green, relative to the small, flat, and tawny unfertilized ovules.

Stigma closure time was estimated prior to the flowers being measured. Forceps were used to contact the stigma and a stopwatch was used to record the length of time taken for lobes to close. All measurements were taken within a two-week period and at the same time of day to reduce environmental variation, which can affect closure speeds (Meinke 1992, Fetscher and Kohn 2001). Closure times were obtained for between 12 and 19 individuals per population.

Individual outcrossing rates could not be estimated because of small seed size and very low seed germination for some populations. Therefore I estimated outcrossing rates for each population by assaying 38.7 individuals on average for four (or 12 for highly inbred populations) enzyme systems. The proteins were extracted with a Tris-HCl, pH 7.5 grinding buffer (Ganders et al. 1985). The enzymes PGI, PGM, 6PGD, and SKDH were run on both morpholine citrate (Ritland and Ganders 1987) and Tris-citrate buffer (system 5, Soltis et al. 1983) systems, to clarify ambiguities in resolution for different enzymes. All enzymes were run on 12.5% starch gels. Staining protocols and recipes were followed from Wendel and Weeden (1989). Because *M. patulus* and *M. ampliatus* had low levels of polymorphism for the above enzymes, I tried eight additional enzymes, MDH, IDH, G6PDH, DIA, and TPI (with Tris-citrate buffer system 5); and ADH, AAT, ACP, with sodium-borate buffer system (system 6, Soltis et al. 1983). No polymorphic loci were resolved for *M. patulus* – 1, and thus outcrossing rates could not be calculated for this population. Recipes are given in Appendix 2-2.

Wright's fixation index (F = 1- observed heterozygosity/expected heterozygosity) was averaged across loci for each population and then outcrossing rate, *t*, was obtained using the relationship:

$$t = (1 - F)/(1 + F)$$

This method assumes equilibrium conditions (Crow and Kimura 1970, Dudash and Fenster 2001), such as outcrossing rates remaining constant among generations (Ritland and Ritland 1989), and no population substructuring. Population-level outcrossing rates are likely an upper limit because F may decrease through a cohort life span due to differential mortality of inbred individuals (Ritland 1996). However, the use of population-based measures of inbreeding history have the advantage over individual selfing rates because they are a cumulative measure over years, integrating other factors leading to inbreeding, and it is therefore less sensitive to yearly variation in selfing rates (Latta and Ritland 1994, Husband and Schemske 1996, Dudash and Fenster 2001).

To test if differences in mating system-traits exist between populations of the same species, Student's *t*-test was employed for the following traits: corolla height, corolla length, pistil length, anther-stigma separation, stigma closure time, pollen and ovule number, P/O, and autonomous seed set. Nested ANOVA (populations nested within species) was employed to test for differences in floral architecture and stigma closure times among species.

Correlations between morphological mating system traits and outcrossing rates (*t*), P/O's, autonomous seed set, were analyzed in two ways, i.e., with and without phylogenetic correction. First, species means in the *M. moschatus* alliance and taxa from the *M. guttatus* complex (Ritland and Ritland 1989) were each treated as independent data points. Second, independent contrasts (Felsenstein 1985) were used to incorporate the phylogenetic structure, including the population data for the *M. moschatus* alliance. The results of the phylogenetically 'naïve' and corrected analyses were compared to interpret the extent of phylogenetic effect and the nature of the phylogenetic signal (Armbruster et al. 2002). To address the problem of phylogenetic structure I used Pagel's (1992) modification of Felsenstein's (1985) analysis of independent contrasts in the

computer program CAIC (Purvis and Rambaut 1995). Figure 2-2 shows the strict-consensus composite tree (i.e., supertree, sensu Bininda-Edmonds and Sanderson 2001), based on two nuclear, two cytoplasmic gene trees (Whittall 1999, Beardsley unpubl.), the allozyme based dendrogram of the *M. guttatus* species complex in Ritland and Ritland (1989), and the 'unbiased' morphological tree for the *M. moschatus* alliance (Carlson unpubl. manuscript). For this analysis, all branch lengths were assumed equal because supertree analysis does not incorporate branch lengths of the original data sets. Populations within the *M. moschatus* alliance were given branch lengths equal to taxa in the *M. guttatus* species complex. Some taxonomists regard the *M. guttatus* species complex as a series of highly polymorphic populations (e.g., Thompson 1993), and very little variation is present in sequenced nuclear and chloroplast genes in this complex (Beardsley, unpubl. data). Therefore, it seems defensible to treat populations in the *M. moschatus* alliance equally with 'species' in the *M. guttatus* species complex. Last, the scaling effects of floral size were evaluated by partial correlation; holding corolla size constant (see Hufford 1988).

RESULTS

Large differences in floral architecture and stigma closure times were found among species in the *M. moschatus* alliance ($P < 0.01$ for all traits except anther-stigma separation, nested ANOVA; Table 2-2), while very little variation was present among populations of the same species (Table 2-3). *Mimulus patulus* has the smallest corolla, shortest style length, no anther-stigma separation, and slow stigma closure. *Mimulus floribundus* has somewhat larger corollas and style lengths, but an even slower stigma closure time. *Mimulus dudleyi*, which is sister to *M. floribundus*, has one of the largest corollas and the greatest anther-stigma distance, but also the slowest stigmatic closure time. The two Columbia River species (*M. jungermannioides* and *M. washingtonensis*; Whittall 1999) have large corolla size, moderately large anther-stigma separation, and very rapid stigma closure times. *Mimulus ampliatus* and *M. hymenophyllus* have

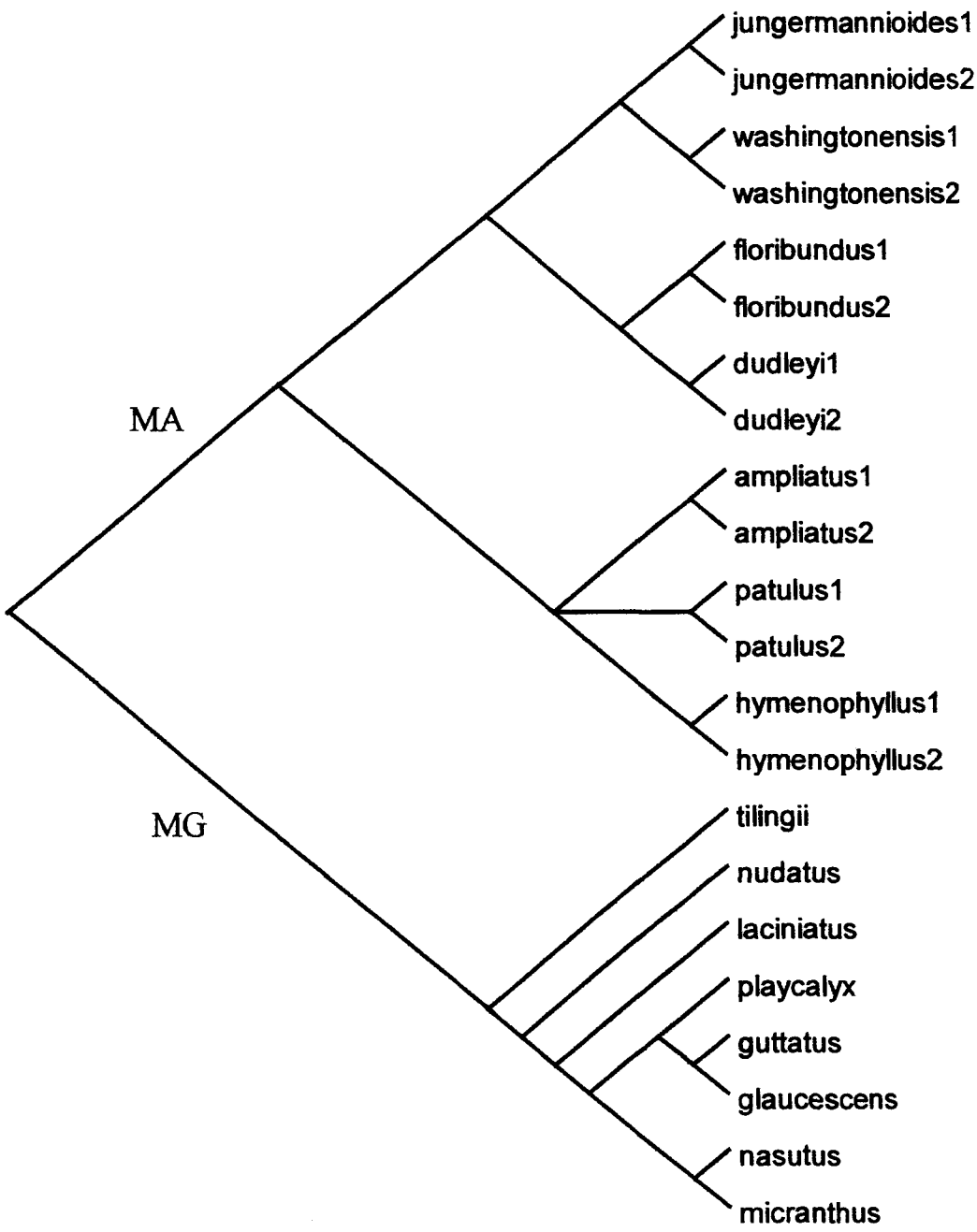

FIGURE 2-2. Strict-consensus supertree of selected members of the *M. moschatus* alliance and the sister clade, *M. guttatus* species complex. Two populations are present for the *M. moschatus* alliance members. MA = *M. moschatus* alliance, MG = *M. guttatus* species complex.

intermediate corolla sizes and stigmatic closure times. *Mimulus hymenophyllus*, *M. jungermannioides*, and to a lesser degree, *M. ampliatus* displayed population-level variation in mating system traits (i.e., corolla length, ovule number, pollen number in *M. jungermannioides*; P/O and autonomous seed set in *M. hymenophyllus*).

Similarly, Ritland and Ritland (1989) observed a wide range of floral architecture in the *M. guttatus* species complex. This species group has some taxa that are morphologically equivalent to strict inbreeders such as *M. floribundus* and *M. patulus*, some intermediate taxa, and some taxa with morphologies associated with greater outcrossing than in the *M. moschatus* alliance. For comparison, anther-stigma separation ranged from –0.21 to +1.10 mm in the *M. moschatus* alliance, and from –0.54 to +2.82 mm in the *M. guttatus* species complex.

Differences in traits more directly associated with mating system also varied among species in the *M. moschatus* alliance. Pollen production differed by a factor of five between *M. patulus* and *M washingtonensis* (Table 2-4). Ovule numbers were less divergent among species, although *M. hymenophyllus* produced substantially fewer ovules than all other species. P/O's ranged from 5.9 to 25.8 among species. Pollen volume was very similar among species, with the exception of *M. hymenophyllus*, which had significantly larger pollen (Argue 1986). The pollen volume of this species was roughly twice that of the other species. The rank of each species in the ratio of male to female gamete investment closely followed P/O rankings. However, *M. hymenophyllus* allocated far less in male/female resources than would be indicated by its P/O, due to substantial investment in seed size. Also, *M. jungermannioides* and *M. washingtonensis* had different P/O and M/F rankings; this was due to relatively larger pollen grains in *M. jungermannioides*.

TABLE 2-2. Floral architecture and stigmatic closure times in seven species in the *M. moschatus* alliance. All measurements are given in mm, except stigma closure time in s. Means are followed by standard errors.

Species	Corolla height	Corolla length	Pistil length	Anther-Stigma separation	Stigma closure time
M. ampliatus	11.38 ± 0.29	16.17 ± 0.25	13.54 ± 0.16	0.66 ± 0.07	3.57 ± 0.63
M. dudleyi	13.61 ± 0.36	15.25 ± 0.25	9.13 ± 0.12	1.10 ± 0.07	9.57 ± 0.43
M. floribundus	8.06 ± 0.22	11.74 ± 0.13	8.91 ± 0.10	-0.14 ± 0.03	7.78 ± 0.65
M. hymenophyllus	10.21 ± 0.23	15.19 ± 0.25	12.53 ± 0.18	0.05 ± 0.05	3.99 ± 0.25
M. jungermannioides	14.02 ± 0.26	16.30 ± 0.21	14.50 ± 0.14	0.41 ± 0.06	2.95 ± 0.17
M. patulus	4.77 ± 0.14	9.36 ± 0.13	7.46 ± 0.09	-0.21 ± 0.03	7.57 ± 0.63
M. washingtonensis	13.05 ± 0.25	14.27 ± 0.16	12.83 ± 0.14	0.81 ± 0.09	1.67 ± 0.12

TABLE 2-3. Floral architecture and stigmatic closure times in 14 populations (seven species) in the *M. moschatus* alliance. All measurements are given in mm, except stigma closure time in s. Means are followed by standard errors. Significant differences (*t*-test) between populations are indicated as* = $p < 0.05$, ** = $p < 0.01$.

Species-Population	Corolla height	Corolla length	Pistil length (stigma position)	Anther-stigma separation	Stigma closure time
M. ampliatus-1	11.81 ± 0.40	16.88 ± 0.34**	13.89 ± 0.23*	0.61 ± 0.09	3.46 ± 0.24
M. ampliatus-2	10.97 ± 0.41	15.50 ± 0.28	13.21 ± 0.21	0.70 ± 0.09	3.67 ± 0.47
M. dudleyi-1	13.51 ± 0.50	15.01 ± 0.31	9.01 ± 0.17	1.08 ± 0.09	9.32 ± 0.41
M. dudleyi-2	13.71 ± 0.56	15.49 ± 0.40	9.23 ± 0.16	1.12 ± 0.08	9.82 ± 0.76
M. floribundus-1	7.92 ± 0.31	11.62 ± 0.17	8.76 ± 0.14	-0.16 ± 0.04	7.70 ± 0.67
M. floribundus-2	8.19 ± 0.32	11.87 ± 0.19	9.05 ± 0.12	-0.13 ± 0.06	7.87 ± 1.13
M. hymenophyllus-1	10.57 ± 0.33	16.07 ± 0.27**	13.20 ± 0.21**	0.13 ± 0.05	3.67 ± 0.40
M. hymenophyllus-2	9.83 ± 0.31	14.27 ± 0.33	11.84 ± 0.22	-0.02 ± 0.08	4.27 ± 0.30
M. jungermannioides-1	13.87 ± 0.43	15.81 ± 0.26**	14.10 ± 0.18**	0.41 ± 0.06	3.04 ± 0.27
M. jungermannioides-2	14.08 ± 0.31	16.75 ± 0.28	14.87 ± 0.16	0.41 ± 0.09	2.87 ± 0.02
M. patulus-1	4.54 ± 0.19	9.51 ± 0.23	7.47 ± 0.15	-0.25 ± 0.03	8.19 ± 0.94
M. patulus-2	4.96 ± 0.19	9.22 ± 0.15	7.46 ± 0.10	-0.17 ± 0.03	6.86 ± 0.81
M. washingtonensis-1	12.93 ± 0.31	14.22 ± 0.21	12.83 ± 0.14	0.88 ± 0.12	1.60 ± 0.14
M. washingtonensis-2	13.20 ± 0.43	14.27 ± 0.24	12.79 ± 0.21	0.77 ± 0.13	1.64 ± 0.20

Autonomous seed set (ratio of autonomously self-fertilized ovules to total ovule number) ranged from 0.028 in *M. washingtonensis* to 0.90 in *M. floribundus*; inbreeding coefficients and outcrossing rates ranged from completely inbred (0.0) to moderately outcrossed (0.64). P/O, M/F, autonomous seed set, and *F* and *t* are given in Table 2-5 for each species; and population statistics for pollen and ovules per flower, P/O, autonomous seed set, and *F* and *t* are given in Table 2-6. Some populations differed substantially in pollen and ovule production; e.g., the two *M. jungermannioides* populations differ in these two measures by about 30 %. These differences in pollen and ovule numbers between populations rarely translated into meaningful differences in P/O's because pollen and ovule numbers tended to covary.

Pollen-ovule ratios were a relatively reliable measure of mating system within each clade and among all members of both the *M. moschatus* alliance and *M. guttatus* species complex (Fig. 2-3, Table 2-7). The relationship was strongly positive in both phylogenetically naïve and corrected (PC) analyses (naïve $r = 0.765$, $p = 0.001$; PC $r = 0.656$, $p = 0.002$). In the naïve analysis and PC analysis, P/O explained 59 % and 43 %, respectively, of the variation in outcrossing rates. The taxa with P/O's of less than ten (between cleistogamy and obligate autogamy, using Cruden's [1977] index) were highly inbred, with outcrossing rates of less than 0.20. The agreement with Cruden's (1977) mating system categories declined in the more outcrossing *Mimulus* taxa, as *Mimulus* P/O's were all under 30, or "obligate autogamous," despite outcrossing rates of near 0.70 in some cases.

Most populations with stigmas at the level of or behind anthers generally experienced very little outcrossing (Fig. 2-3). However, both populations of *M. hymenophyllus* maintained a moderate level of outcrossing despite no anther-stigma separation. Conversely, *M. ampliatus* had moderately large anther-stigma separation, but very little outcrossing.

TABLE 2-4. Pollen and ovule numbers per flower and pollen and seed volume in seven species in the *M. moschatus* alliance. Values for pollen and seed volume (μm^3) are calculated in part from Argue (1986), see text for details. Means are followed by standard errors for the counts.

Species	Pollen no.	Pollen vol.	Ovule no.	Seed vol.
M. ampliatus	3,833 ± 244	78.5×10^3	323.2 ± 20.6	22.0×10^6
M. dudleyi	5,843 ± 439	78.5×10^3	386.9 ± 23.5	19.7×10^6
M. floribundus	2,721 ± 207	74.8×10^3	284.8 ± 16.9	19.7×10^6
M. hymenophyllus	3,131 ± 139	167.4×10^3	122.9 ± 7.9	64.0×10^6
M. jungermannioides	3,965 ± 286	104.4×10^3	208.2 ± 12.0	17.6×10^6
M. patulus	1,078 ± 96	83.5×10^3	186.0 ± 12.5	15.6×10^6
M. washingtonensis	4,876 ± 294	90.6×10^3	248.7 ± 12.7	19.7×10^6

TABLE 2-5. Mating system indicators in seven species in the *M. moschatus* alliance. Pollen-ovule (P/O) ratios, index of male/female gamete investment (M/F = 1000 X [pollen no. X pollen vol.]/[ovule no. X seed vol.]), greenhouse autonomous seed set, Wright's inbreeding coefficient (*F*) and outcrossing rate (*t*). Means are followed by standard errors. Autonomous seed set was arcsine square-root transformed to meet normality assumptions.

Species	P/O	M/F	Auto Seed Set	F & *t*
M. ampliatus	12.24 ± 0.53	42.4	0.52 ± 0.05	0.94, 0.03
M. dudleyi	15.70 ± 0.80	60.2	0.59 ± 0.05	0.39, 0.44
M. floribundus	8.48 ± 0.42	36.3	0.90 ± 0.02	(0.99, 0.01)
M. hymenophyllus	25.81 ± 1.22	66.6	0.70 ± 0.03	0.41, 0.42
M. jungermannioides	19.70 ± 0.86	113.0	0.18 ± 0.03	0.46, 0.38
M patulus	5.91 ± 0.30	31.0	0.89 ± 0.02	0.84, 0.09
M. washingtonensis	20.34 ± 0.81	90.2	0.028 ± 0.02	0.22, 0.64

TABLE 2-6. Mating system indicators in 14 populations in the *M. moschatus* alliance. Pollen-ovule (P/O) ratios, greenhouse autonomous seed set, Wright's inbreeding coefficient (F) and outcrossing rate (*t*), and pollen and ovule numbers per flower. Means are followed by standard errors. Significant differences (*t*-test) between populations are indicated as* = $p < 0.05$, ** = $p < 0.01$. Autonomous seed set was arcsine square-root transformed to meet normality assumptions.

Species	Pollen no.	Ovule no.	P/O	Auto Seed Set	F & *t*
M. ampliatus-1	3,966 ± 367	368.9 ± 32.0	11.87 ± 0.80	0.52 ± 0.08	1.0, 0.0
M. ampliatus-2	3,667 ± 313	280.0 ± 22.7	12.71 ± 0.66	0.51 ± 0.08	0.88, 0.06
M. dudleyi-1	5,820 ± 627	399.1 ± 31.4	14.79 ± 0.91	0.64 ± 0.07	0.41, 0.41
M. dudleyi-2	5,865 ± 671	375.1 ± 35.2	16.55 ± 1.28	0.55 ± 0.08	0.36, 0.47
M. floribundus-1	2,821 ± 337	281.3 ± 23.5	8.97 ± 0.73	0.88 ± 0.04	1.0, 0.0
M. floribundus-2	2,633 ± 259	288.1 ± 24.7	8.05 ± 0.45	0.92 ± 0.03	0.98, 0.01
M. hymenophyllus-1	3,122 ± 187	141.1 ± 10.5**	23.12 ± 1.46**	0.59 ± 0.03**	0.39, 0.44
M. hymenophyllus-2	3,140 ± 233	108.1 ± 10.6	28.09 ± 1.72	0.80 ± 0.05	0.43, 0.40
M. jungermannioides-1	3,356 ± 303**	170.0 ± 12.8**	20.37 ± 1.33	0.23 ± 0.05	0.54, 0.30
M. jungermannioides-2	4,665 ± 423	252.9 ± 16.7	18.83 ± 1.01	0.14 ± 0.04	0.38, 0.45
M. patulus-1	1,076 ± 143	172.0 ± 16.0	5.96 ± 0.41	0.89 ± 0.03	-
M. patulus-2	1,081 ± 135	200.1 ± 19.2	5.86 ± 0.45	0.89 ± 0.03	0.84, 0.09
M. washingtonensis-1	4,992 ± 478	252.1 ± 17.3	20.80 ± 1.31	0.05 ± 0.03	0.25, 0.60
M. washingtonensis-2	4,765 ± 383	242.4 ± 19.9	20.05 ± 1.06	0.005 ± 0.003	0.19, 0.69

Autonomous seed set was strongly negatively correlated with outcrossing rate (r = -0.713, $P < 0.01$; Fig. 2-3, Table 2-7). This relationship was also suggested within species, as in five of six species, the population with a greater outcrossing rate also had lower autogamous seed set in the greenhouse. The ability of populations to set seed without visitation was clearly reflected in field outcrossing rates, although these two traits can be decoupled under intensive visitation.

High outcrossing rates were often accompanied by greater male resource allocation. The function of the corolla has been suggested to be largely male (Charlesworth and Charlesworth 1981, Ritland and Ritland 1989). Corolla size was indeed positively related to outcrossing rates, male/female resource investment, pollen number, and anther-stigma separation in the *M. moschatus* alliance (Fig. 2-4, Fig. 2-5, Table 2-7). Interestingly, many floral traits did not display significant scaling effects associated with corolla size. After controlling for corolla size (not shown), anther-stigma separation and pollen number were two traits that showed substantial reductions in the strength of their relationship with outcrossing rates.

Male relative to female resource investment (M/F) should be a better estimate of sex-based resource investment than P/Os, since the sex-specific energetic costs can differ significantly among *Mimulus* species (see Argue 1986), and pollen and seed volume should approximate energetic costs. Like P/O, M/F was positively related to outcrossing rates, but the correlation was significant only in the naïve analysis (Fig. 2-5, Table 2-7).

Pollen volume has been suggested to be positively associated with outcrossing (Barret, Harder, and Woley 1997), however, this relationship was not supported in these analyses (Fig. 2-4, Table 2-7). Pollen volume was positively correlated with P/O in all analyses, while no relationship was apparent between pollen volume and pollen number.

I found no evidence that stigma closure time is an outcrossing mechanism. Stigma closure time was not strongly correlated with any other trait in this study (Table 2-7).

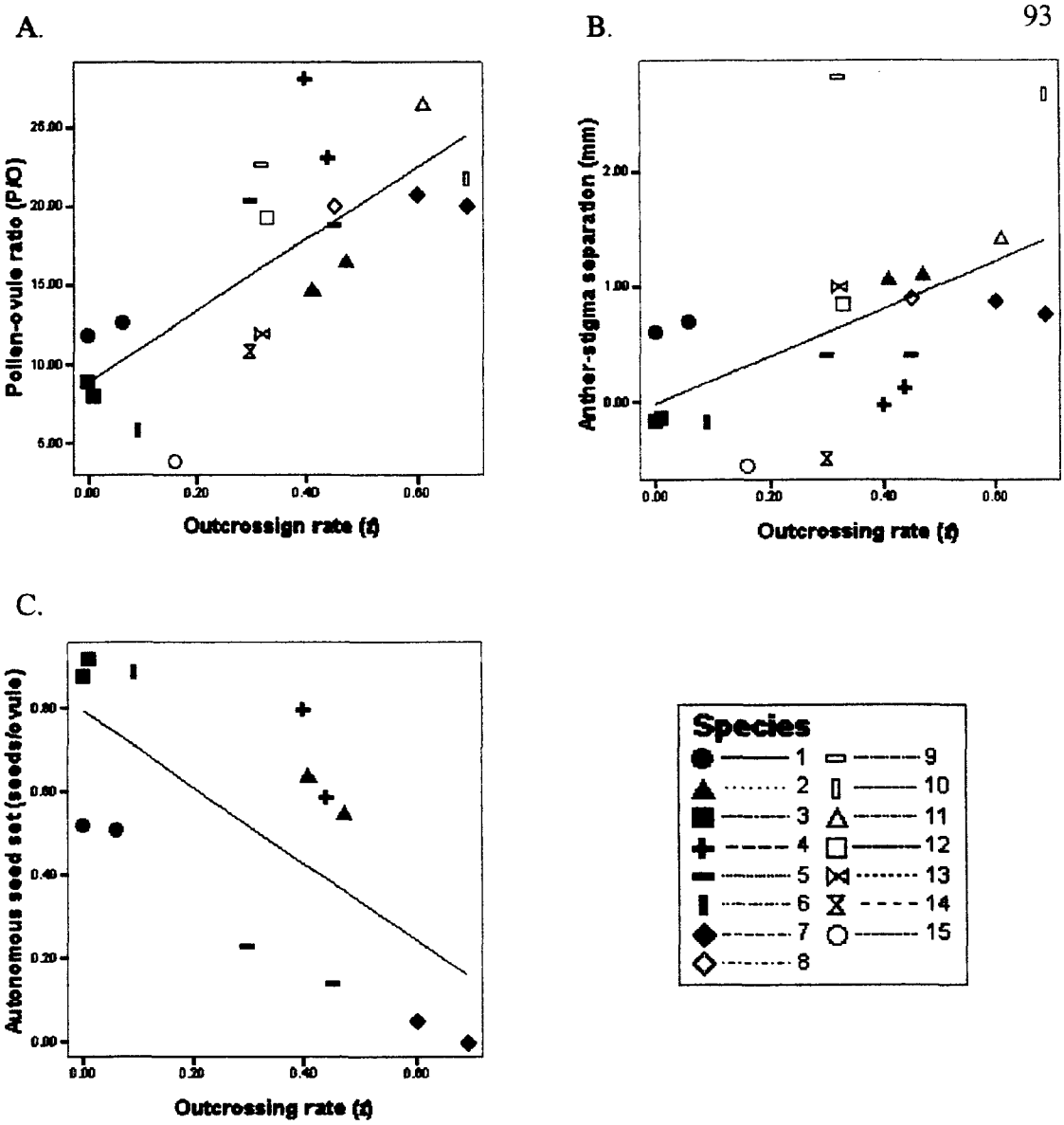

FIGURE 2-3. The relationship between outcrossing rate and (A) pollen-ovule ratio, (B) anther-stigma separation, and (C) autonomous seed set. Population in the *M. moschatus* alliance share filled symbols: 1 = *M. ampliatus*, 2 = *M. dudleyi*, 3 = *M. floribundus*, 4 = *M. hymenophyllus*, 5 = *M. jungermannioides*, 6 = *M. patulus*, 7 = *M. washingtonensis*. Taxa from the *M. guttatus* species complex (Ritland and Ritland 1989) are indicated as open symbols: 8 = *M. guttatus*, 9 = *M. nasutus*, 10 = *M. glaucescens*, 11 = *M. tilingii*, 12 = *M. nudatus*, 13 = *M. laciniatus*, 14 = *M. platycalyx*, 15 = *M. micranthus*.

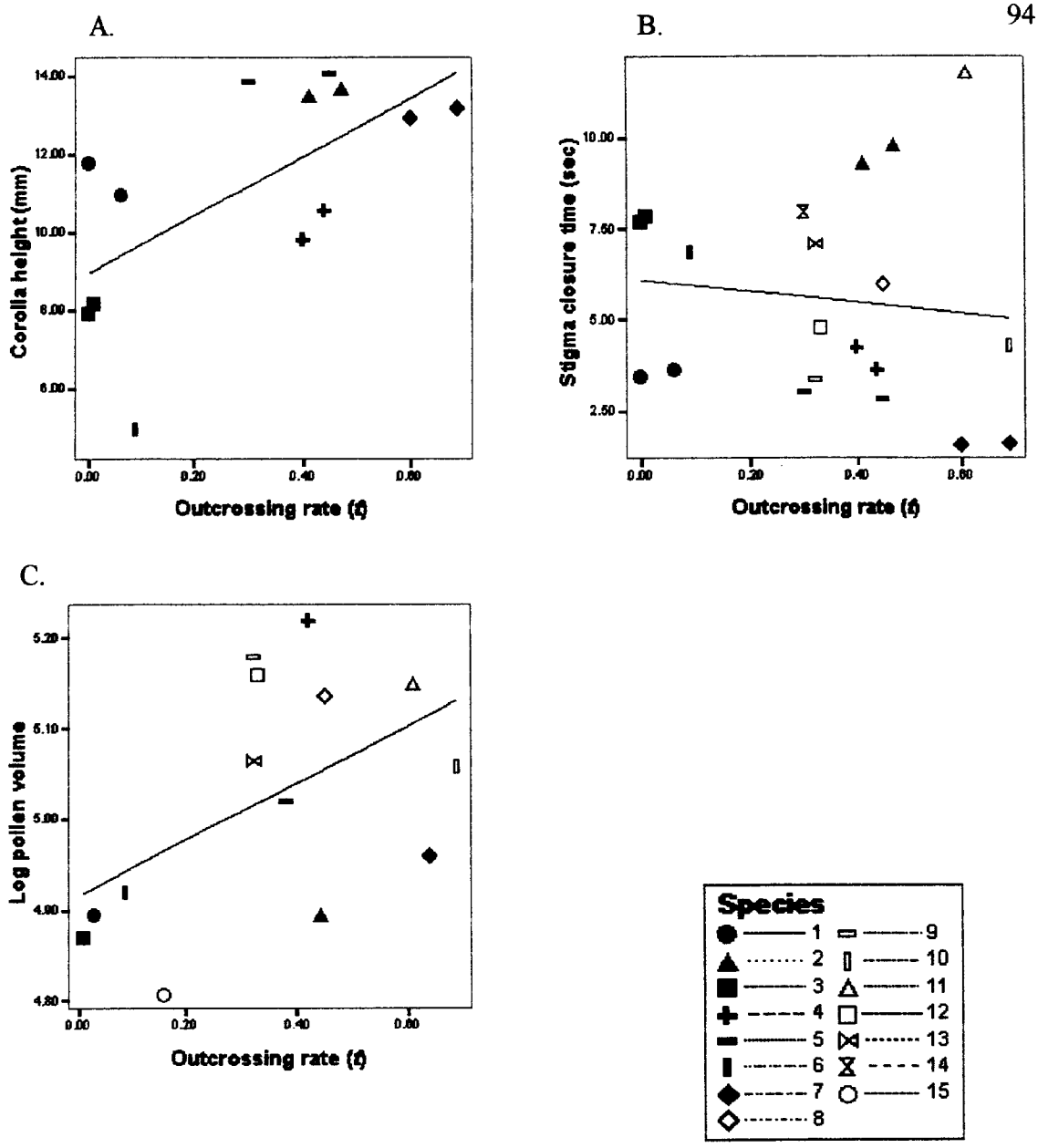

FIGURE 2-4. The relationship between outcrossing rate and (A) corolla height, (B) stigma closure time, and (C) pollen volume. Population in the *M. moschatus* alliance share filled symbols: 1 = *M. ampliatus*, 2 = *M. dudleyi*, 3 = *M. floribundus*, 4 = *M. hymenophyllus*, 5 = *M. jungermannioides*, 6 = *M. patulus*, 7 = *M. washingtonensis*. Taxa from the *M. guttatus* species complex (Ritland and Ritland 1989) are indicated as open symbols: 8 = *M. guttatus*, 9 = *M. nasutus*, 10 = *M. glaucescens*, 11 = *M. tilingii*, 12 = *M. nudatus*, 13 = *M. laciniatus*, 14 = *M. platycalyx*, 15 = *M. micranthus*.

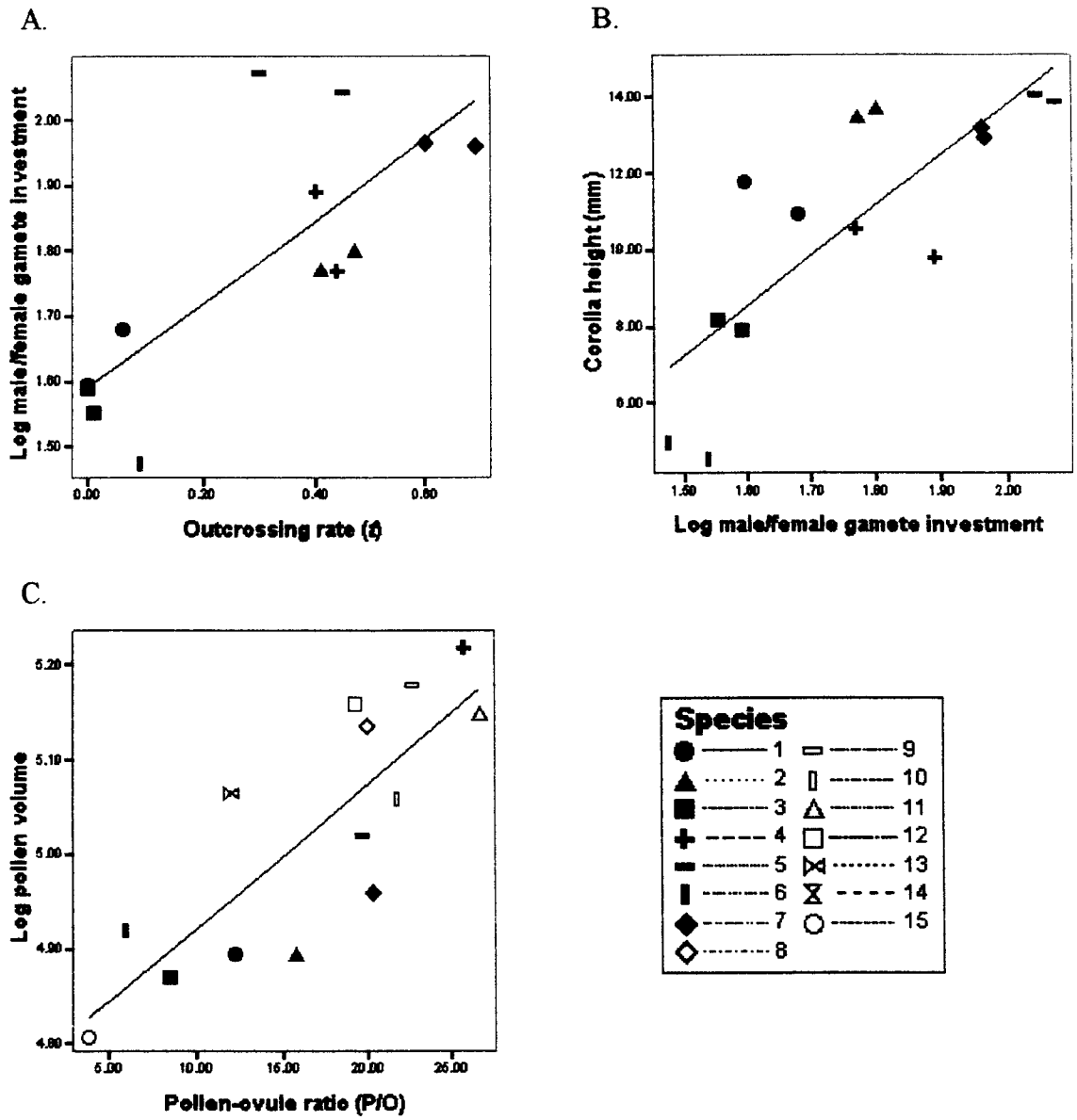

FIGURE 2-5. Sex allocation relationships. (A) = outcrossing rate and male/female gamete investment, (B) = M/F and corolla height, (C) = P/O and pollen volume. Population in the *M. moschatus* alliance share filled symbols: 1 = *M. ampliatus*, 2 = *M. dudleyi*, 3 = *M. floribundus*, 4 = *M. hymenophyllus*, 5 = *M. jungermannioides*, 6 = *M. patulus*, 7 = *M. washingtonensis*. Taxa from the *M. guttatus* species complex (Ritland and Ritland 1989) are indicated as open symbols: 8 = *M. guttatus*, 9 = *M. nasutus*, 10 = *M. glaucescens*, 11 = *M. tilingii*, 12 = *M. nadutus*, 13 = *M. laciniatus*, 14 = *M. platycalyx*, 15 = *M. micranthus*.

TABLE 2-7. Correlations of mating system indicators among seven species of *Mimulus*. When eight additional taxa from the *M. guttatus* species complex are included (Ritland and Ritland 1989) values are indicated in parentheses ($n = 15$). Phylogenetically corrected correlations within the *M. moschatus* alliance include two populations per species, and are represented below the diagonal; $n = 11$ contrasts, and in parentheses among the *M. moschatus* alliance and *M. guttatus* species complex combined; $n = 18$ contrasts. (** = P < 0.01, * = P < 0.05, † = P < 0.10; two tailed test).

	t	P/O	Male/ Female Investment	Log pollen number	Log pollen volume	Log seed volume
t	-	0.792* (0.765**)	0.758*	0.584 (0.629*)	0.435 (0.550†)	0.237
P/O	0.652* (0.656**)	-	0.738*	0.591 (0.712**)	0.796* (0.825**)	0.679†
Male/Female Investment	0.481	0.801**	-	0.582	0.402	0.031
Log pollen Number	0.845** (0.354)	0.432 (0.705**)	0.361	-	0.022 (0.516†)	0.131
Log pollen Volume	0.311 (0.288)	0.662* (0.614**)	0.594†	0.148 (0.272)	-	0.825**
Log seed Volume	-0.033	0.311	-0.154	-0.166	0.545†	-
Seed set	-0.570†	-0.343	-0.549*	-0.347	-0.262	0.379
Corolla height	0.707*	0.526†	0.680*	0.755**	0.474	-0.251
Anther-stigma	0.780** (0.551*)	0.329 (0.576*)	0.225	0.722** (0.516*)	0.109 (0.085)	-0.250
Stigma closure time	-0.091 (-0.006)	-0.210 (-0.132)	-0.279	0.284 (0.068)	-0.244 (-0.194)	-0.482

TABLE 2-7, continued. Correlations of mating system indicators among seven species of *Mimulus*.

	t	Seed set	Corolla height	Anther-stigma separation	Stigma closure time
t	-	-0.713†	0.660	0.559 (0.559*)	-0.394 (-0.062)
P/O	0.652* (0.656**)	-0.574	0.660	0.348 (0.629*)	-0.585 (-0.223)
Male/Female Investment	0.481	-0.865*	0.797*	0.461	-0.606
Log pollen Number	0.845** (0.354)	-0.640	0.940**	0.847* (0.747**)	-0.266 (-0.069)
Log pollen Volume	0.311 (0.288)	-0.117	0.113	-0.227 (0.507†)	-0.431 (-0.113)
Log seed Volume	-0.033	0.149	0.050	-0.156	-0.237
Seed set	-0.570†	-	-0.795*	-0.642	0.743†
Corolla height	0.707*	-0.596†	-	0.833*	-0.408
Anther-stigma	0.780** (0.551*)	-0.403	0.713*	-	-0.139 (-0.174)
Stigma closure time	-0.091 (-0.006)	-0.343	0.102	0.261 (-0.233)	-

Outcrossing rates and morphological traits associated with mating system all varied continuously. Taxa in the *M. moschatus* alliance and *M. guttatus* complex ranged, without substantial gaps, from highly inbred to moderately outcrossed, as reflected in outcrossing rates and P/O's (Fig. 2-6).

Taking phylogeny into account did not alter the results greatly. The relationships that were strongly influenced by phylogeny were between M/F and outcrossing rates, and between corolla height and autonomous seed set. Further, trait relationships were similar, and generally stronger, when data from the *M. guttatus* complex were included. Differences between populations of the same species were relatively uninformative relative to those among species and among higher nodes in the independent contrasts. One exception to this was a relatively large difference in P/O between the two populations of *M. hymenophyllus*. However, the difference in P/O was not coupled with large differences in other mating system traits. Figure 2-7 shows independent contrasts between P/O and outcrossing rate, autonomous seed set, stigma closure time, and corolla height with contrasts between populations and among higher phylogenetic nodes noted.

DISCUSSION

Reproductive morphology varies greatly among even closely related species in the genus *Mimulus*, a pattern observed widely across flowering plants (Ornduff 1969, Wyatt 1983, Ritland and Ritland 1989, Dole 1992). This variation in morphology is often correlated with outcrossing rates (Schoen 1982a, Ganders et al. 1985, Barrett and Husband 1990, Belaoussoff and Shore 1995, Brunet and Eckert 1998). As in many groups, the *M. moschatus* alliance has small-flowered, highly selfing species that are very closely related to larger flowered, more outcrossing species. In addition, this group contains members

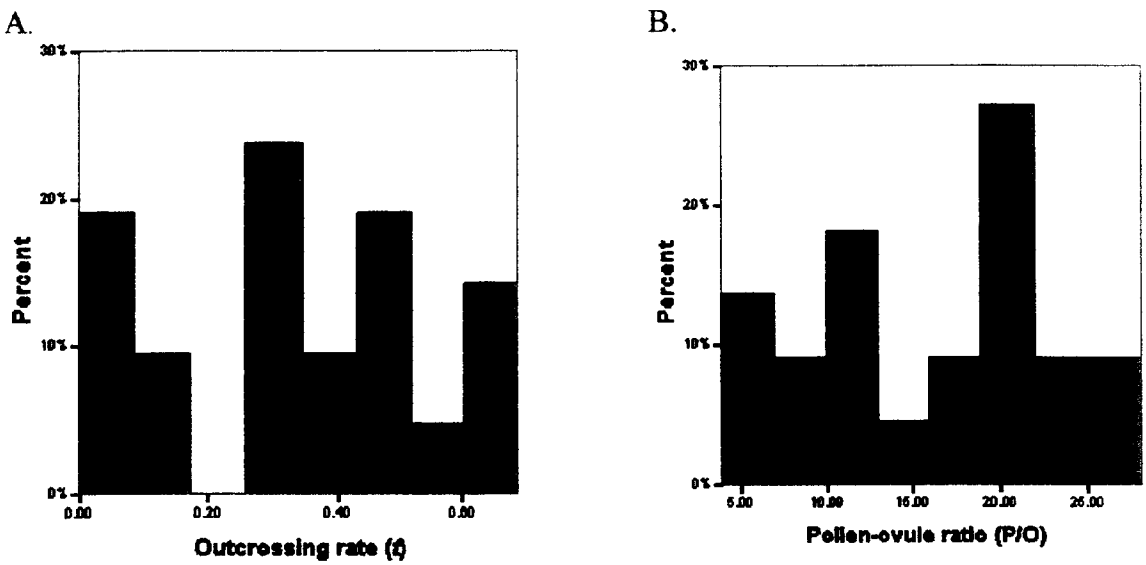

FIGURE 2-6. Frequency distribution of (A.) outcrossing rates and (B.) P/O's among 15 species (20 populations total) in the *M. moschatus* alliance and *M. guttatus* complex.

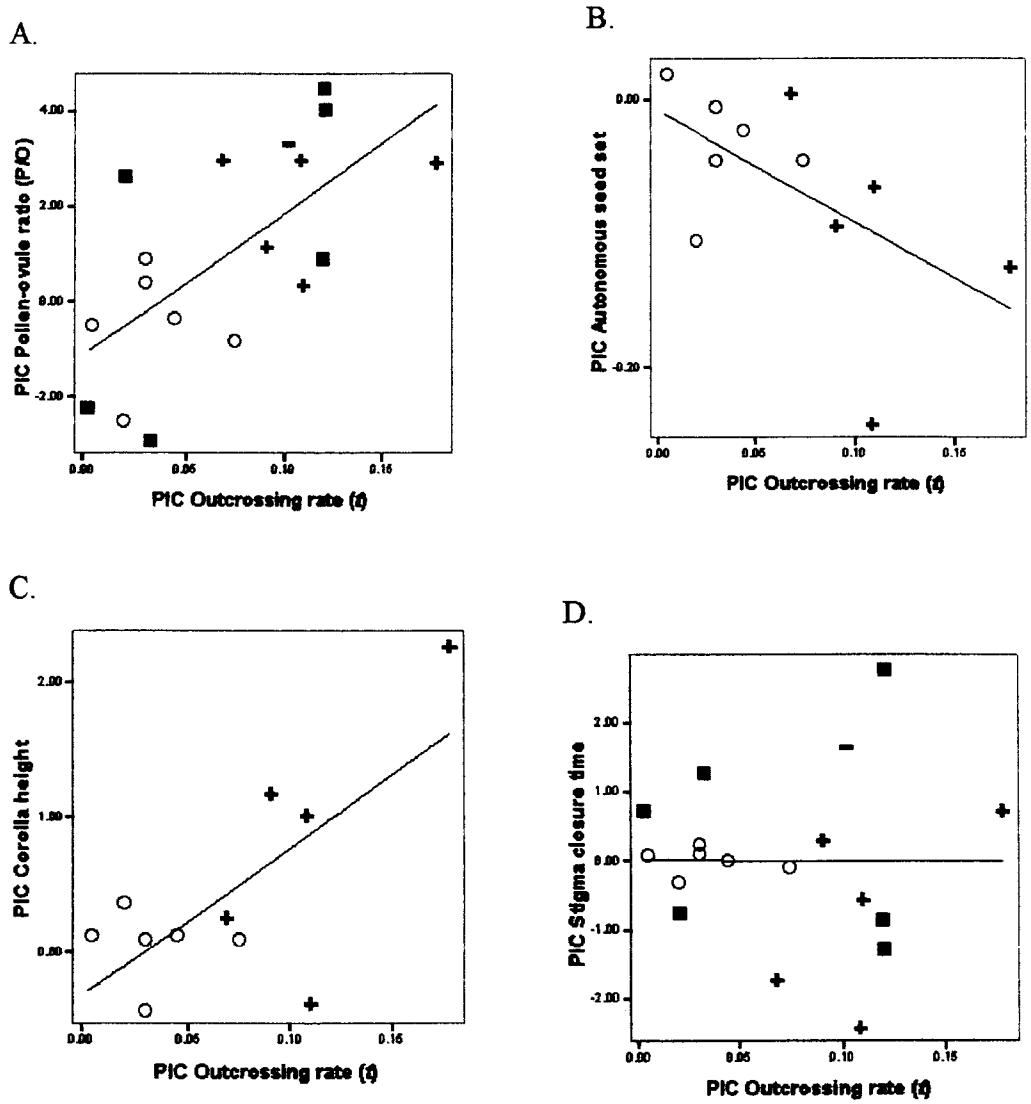

FIGURE 2-7. Phylogenetic independent contrasts (PIC) between outcrossing rate (*t*) and (A) pollen-ovule ratio, (B) autonomous seed set, (C) corolla height, and (D) stigma closure time. Between population contrasts are indicated as circles, among species and higher node contrasts within the *M. moschatus* alliance are indicated as plusses, among the *M. guttatus* complex as squares, and between the two species complexes as a minus.

with intermediate mating systems. Unlike in *M. guttatus* (Dole 1992), populations within species differed very little in morphology and in outcrossing rates, as estimated from allozyme data.

Two species in the *M. moschatus* alliance, *M. patulus* and *M. floribundus*, are highly autogamous. These species had very low outcrossing rates and possessed floral morphologies consistent with an autogamous mating system. At the time of anthesis there was no anther-stigma separation and these two species were not protogynous, unlike the other members of the *M. moschatus* alliance (however, the period of protogyny is generally only a few hours for most species in the alliance). The corollas of *M. patulus* and *M. floribundus* are very small, although not cleistogamous, and ca. 90 % of ovules were shown to self-fertilize without any floral visitation.

Mimulus ampliatus and *M. hymenophyllus* have an autogamous to mixed-mating system, with intermediate corolla size, autonomous seed set, anther-stigma separation, and allozyme-estimated outcrossing rate. The outcrossing rate for *M. ampliatus* was actually much lower than expected based on its floral morphology and is likely the result of biparental inbreeding. Populations of this species are small, isolated, and ruderal, suggesting that stochastic genetic processes were likely responsible for a high degree of biparental inbreeding. This species also displayed some population-level variation in a number of morphological traits, supporting the possibility of strong genetic drift.

The most outcrossing species in the alliance are *M. jungermannioides*, *M. dudleyi*, and *M. washingtonensis*. These species are best characterized as having a mixed mating system, as outcrossing rates ranged from 0.38 to 0.64. All species analyzed were self compatible, had a delayed selfing mechanism, and multiple flowers per plant were open at any one time, so that outcrossing rates would not be expected to approach 1.0, even under conditions of high floral visitation. These species all had the largest corollas, anther-stigma separation, and P/Os. Additionally, the outcrossing species had the greatest floral longevity (ca. 3-4 d) and protogynous period (up to ca. 12 h in *M. washingtonensis*).

For most species, the two populations examined were not significantly different in mating system traits; however, three species with very isolated populations showed some differences. *Mimulus ampliatus* and *M. jungermannioides* each had one population with longer corollas, pistils, and more ovules. Additionally, one population of *M. jungermannioides* had 1.5 times the number of pollen grains as the other population. *Mimulus hymenophyllus* had one population with longer corollas, pistils, more ovules, smaller P/Os and autonomous seed set. The larger-flowered populations of each species had greater outcrossing rates; however, with the exception of *M. jungermannioides*, the differences in outcrossing rates were minimal. Generally, the traits that showed population-level variation appeared to be overall size-related traits that were not as closely associated with outcrossing rates as P/Os, pollen number, and anther-stigma separation.

Trait covariation and test of predictions –
Correlation analysis was used to assess the covariation between mating system traits in the *M. moschatus* alliance separately and collectively with the *M. guttatus* complex. The relationships were quite similar when including either one or both species groups. In some cases, the relationships were stronger when including the *M. guttatus* complex, and in other cases they were weaker. This indicated that patterns within each group were consistent for only a subset of traits.

Phylogenetic effects – The two data sets (*M. moschatus* alliance and *M. moschatus* alliance + *M. guttatus* complex) were analyzed to address the effect of phylogenetic history. In the naïve analysis, the mean for each species was treated as an independent data point, while in the phylogenetically corrected analysis, which included population data, phylogenetic relationships among species were incorporated into the analysis (see Schwilk and Ackerly 2001, Armbruster et al. 2002). Both analyses gave similar results, suggesting that most morphological traits associated with mating system are evolutionarily labile and not constrained by phylogenetic inertia. However, in some

cases the effect of removing *M. guttatus* complex data resulted in much stronger and statistically significant relationships, suggesting that individual clades have different trait associations. As in tribe *Collinsieae* (Armbruster et al. 2002), the pattern of sister species with divergent flower sizes (e.g., *M. floribundus* and *M. dudleyi*) is consistent with the interpretation of minimal phylogenetic effect. 'Phylogenetic inertia' (and pseudoreplication) is indicated when only the naïve analysis detects significant results. This is due, entirely, to the influence of ancestral character states (Armbruster et al. 2002). When PC analysis detects relationships not apparent in naïve analysis, a 'phylogenetic lag' is suggested, reflecting the influence of both ancestral character states and selection on current phenotypes (Armbruster et al. 2002).

Pollen:ovule ratios – The ratio of pollen number to ovule number has been used as an estimator of mating system (Cruden 1977, Preston 1986). I found P/Os for members of the *M. moschatus* alliance to range from between 5 and 28 (i.e., between cleistogamy and obligate autogamy in Cruden's index; 1997). While Cruden's number index (1997) did not relate well with outcrossing rates in the *M. moschatus* alliance and *M. guttatus* species complex, P/Os were a relatively reliable measure of mating system, as they were closely correlated with outcrossing rates. However, the use of P/Os as a mating system proxy in comparison with more distantly related species is dubious. In a cleistogamous flowered European *Astragalus*, mean P/O was 84 (Gallardo et al. 1994), roughly three times greater than the most outcrossing *Mimulus* species. Likewise, in a survey of 66 Cruciferae taxa (Preston 1986), the autogamous species had very high P/Os relative to *Mimulus*: values typically ranged from 100 to 1000. *Mimulus* have much lower P/Os than many other groups, likely due to androecium structural constraints of only four anthers and/or low pollen loss to inefficient floral visitors, whose numbers are reduced due to zygomorphy and the "semi-protected" anthers of the genus. Low P/Os in *Mimulus* are also a function of high ovule numbers. The characteristic of many small ovules of the gynoecium is rather uniform across *Mimulus* species, and it may be an adaptation to the largely annual habit (however, this relationship is not present in the related genus,

Collinsia, Armbruster pers. comm.). Although, in *M. hymenophyllus* capsule size and ovary number is greatly reduced. This species was also shown to have the greatest P/O, despite having an intermediate outcrossing rate and floral morphology. While pollen numbers were average, ovule numbers were very low, roughly 50% of the other members of the *M. moschatus* alliance. With the decrease in ovule number, seed volume was apparently greatly increased, so that relative male to female resource investment was found to be close to species with similar outcrossing rates. This species dwells in shaded cliff habitats and fewer ovules per flower may be an adaptation to increasing the probability of seeds being deposited into the limited, and patchily distributed safe sites, by increasing the total number of maturing fruits while decreasing individual brood size. Reducing ovule number may also be an adaptive tradeoff for increasing competitive and resource acquisition ability in seedlings by increasing juvenile resource investment. Overall, however, relative P/Os represented a reliable indicator of mating system within these species groups.

Herkogamy – As in many species (e.g., Belaoussoff and Shore 1995, Brunet and Eckert 1998), anther-stigma separation was strongly correlated with outcrossing rate in the *M. moschatus* alliance, supporting the second prediction. Species with no separation (i.e., zero or less) outcrossed very little. However, *M. hymenophyllus* maintained a moderate level of outcrossing despite no anther-stigma separation. This species has a short (2 – 6 hr) protogynous phase, but insect visitation was not common in the cool, shaded cliff habitats (pers. observation), so the moderately high outcrossing rate could be an artifact of higher mortality among homozygous offspring (Ritland 1996). Despite moderately large anther-stigma separation, very little outcrossing was observed in both populations of *M. ampliatus*. Populations of this species were small, isolated, and ruderal, suggesting that stochastic genetic processes were responsible for substantial biparental inbreeding. Anther-stigma separation covaried closely with corolla size. When partial correlation analysis was conducted on anther-stigma separation, after controlling for corolla size, no relationships were apparent. It was therefore difficult to assess the individual influence

of each of these traits on outcrossing rates. Similar relationships were observed in *Eucnide* (Loasaceae: Hufford 1988) and in *Dalechampia* (Euphorbiaceae: Armbruster 1988, 1993) anther-stigma separation covaried with overall blossom size, pollinator size, and autonmous seed set. Strong positive genetic correlations were associated with floral size traits in two populations of *M. guttatus* and *M. micranthus*; however, anther-stigma separation was not correlated with corolla size (Carr and Fenster 1994). This suggests that corolla size and anther-stigma separation can respond individually to selective pressures (see Hansen et al. submitted), and that large corolla size is not simply a pleiotropic response to selection for greater anther-stigma separation (or vice versa).

Autonomous seed set – The ability to auto-fertilize in the absence of pollinators (autonomous seed set) has been employed as a mating system proxy (e.g., Armbruster 1993, Gallardo et al. 1994), despite the argument than autonomous seed set reflects the potential maximum degree of self-fertilization, rather than the actual (Lloyd and Schoen 1992). Under conditions of high pollen flow, especially if selfing is delayed, outcrossing rates may be greater than indicated by autonomous seed set (Kalisz et al. 1999, Armbruster et al. 2002). I predicted that autonomous seed set would be negatively correlated with outcrossing rates. Indeed, in the *M. moschatus* alliance, autonomous seed set was strongly negatively related with outcrossing rates in both the naïve and PC analyses ($r = -0.71$, $p < 0.10$ and $r = -0.57$, $p < 0.10$, naïve and PC analyses, respectively). Thus, pollinators were clearly active during the unisexual phase of the more outcrossing species, while very few pollinators mediated cross pollination in the more autogamous species. The negative relationship between autonomous seed set and outcrossing rate was also illustrated at the within species-level. For most population-pairs, the population with a greater outcrossing rate also had reduced autogamous seed set in the greenhouse.

The mechanism behind autonomous seed set is more involved than simply lack of anther-stigma separation, as these traits are only weakly related to one another. The ability to auto-fertilize in other *Mimulus* taxa is a function of degree of proximal stigma

lobe curling and corolla abscission, in addition to anther-stigma separation (Dole 1990, 1992).

Sex allocation and mating system – The correlation between mating system and many floral traits has been interpreted in the context of sex allocation theory, where investment in male function declines relative to female function as self-fertilization increases (Charnov 1979, Charlesworth and Charlesworth 1981, Charnov 1982). I therefore hypothesized that higher outcrossing rates would accompany increased allocation to male function, i.e., outcrossing rates would be positively correlated with corolla size, pollen number and volume, and male/female resource investment.

The corolla has been argued to have a predominantly male function, as only a single pollinator visit is normally adequate to fertilize all ovules, but multiple pollinator visits are required to remove pollen and disperse it to other plants (Bell 1985). Corolla height in the *M. moschatus* alliance strongly covaried with outcrossing rates, as predicted. Corolla dimensions of the selfing species were 35 to 50% the size of the more outcrossing species. Similarly, in the *M. guttatus* complex, selfing taxa allocated roughly half of floral biomass to male function (corolla + stamens) relative to the outcrossing taxa (Ritland and Ritland 1989). Additionally in the *M. moschatus* alliance, corolla size was positively correlated with male/female resource investment and pollen number, traits clearly associated with male allocation. These results support the link between outcrossing and increased male allocation.

It has been stressed that P/Os may not accurately reflect resource allocation to pollen and ovules, since P/Os do not include resources invested per pollen grain and per ovule (Charnov 1982, Queller 1984). I therefore approximated male and female resource investment (M/F) by multiplying the volume of pollen and seeds by their respective numbers to achieve a better estimate of sex-based resource investment than P/Os. While seeds include both male and female gametic resources, energetically they are a female expense and are therefore treated as ovule energetic cost (see Charlesworth and Charlesworth 1981, Charnov 1982). Volume of pollen was used to approximate

energetic costs, however volume may not accurately reflect costs. Pollen volume was clearly not correlated with pollen biomass (a better estimate of energetic costs) in a group of 22 *Solanum* species (Mione and Anderson 1992). In this analysis, M/F was positively related to outcrossing rates, but the correlation was significant only in the naïve analysis.

Cruden and Miller-Ward (1981) suggested that P/O and pollen grain size should be inversely related because large grains contain greater amounts of enzymes, zymogens, and other compounds needed to facilitate germination and pollen tube growth on a stigma. In species with smaller grains, a greater number of grains is necessary to yield the critical amount of compounds to allow pollen germination and growth. Charnov (1982) hypothesized that pollen number and volume should also vary inversely, when ovule size and breeding system are invariant. This relationship is due to an investment tradeoff. Additionally however, pollen volume has been suggested to be positively associated with outcrossing (Barret, Harder, and Worley 1997), which is normally accompanied by greater pollen numbers. In this analysis, pollen volume was positively correlated with P/O in all analyses. An opposite result was obtained in an analysis of 19 species, encompassing ten families, by Cruden and Miller-Ward (1981). In the *M. moschatus* alliance, no tradeoff was apparent between pollen volume and pollen number. The lack of relationship may reflect a conflict between selection on total pollen investment (both number and volume) and a moderate genetic constraint between pollen number and volume. Likewise, no evidence of a tradeoff between pollen number and size was detected in species of *Arenaria* (Wyatt 1984b). In *Astragalus* subgenus *Epiglottis*, however, a strongly negative and nearly-significant correlation was detected between P/O and pollen volume in a partial correlation analysis, despite very low sample size (Gallardo et al. 1994). This analysis after controlling for ovule size and outcrossing rate (approximated by autonomous seed set) supported the prediction made by sex allocation theory. In the *M. moschatus* alliance, when seed size and outcrossing rates were held constant, the correlation between P/Os and pollen volume was weak and in the opposite direction (not shown).

Stigma closure – Rapic stigmatic closure has been hypothesized to be an outcrossing mechanism. Upon pollinator entry into the corolla a rapidly closing stigma would reduce the amount of self-pollen deposited on the stigma because little receptive surface is available as an insect exits the basal portion of the flower, covered in self-pollen (Ritland and Ritland 1989, Meinke 1992, Fetscher and Kohn 1999). I therefore predicted stigma closure rates would be negatively correlated with outcrossing rates. However, in this analysis stigma-closure time was not strongly correlated with any measure of mating system. The relationships became even weaker when data from the *M. guttatus* species complex were included, as some very large-flowered, outcrossing taxa (e.g., *M. tilingii*) have some of the slowest stigmatic closure times. Phylogenic history may help to explain variation in stigmatic closure time. In the naïve analysis, the correlation between stigma closure time and autonomous seed set was moderately positive and approached significance with a small sample size ($n = 7$). This suggests that evolution of slow stigma closure time may be an adaptation for autonomous seed set rather than fast stigma closure being an outcrossing mechanism. However, after controlling for phylogeny, no relationship was detected (although not significant, the correlation direction was actually reversed), because stigma closure time possesses a large degree of phylogenetic inertia. Related species had very similar stigma closure times, regardless of mating system. Thigmotropic stigmas have been proposed to aid in pollen capture and reducing interference between pollen receipt and export functions (see review in Fetscher and Kohn 1999). However, stigma closure did not improve pollen capture, germination, and fertilization in *M. aurantiacus*, and the pattern observed best fits the hypothesis of reduced interference between male and female function (Fetscher and Kohn 1999). Clearly, more experimentation is required to evaluate the adaptive significance of this behavior.

Frequency distribution of mating system traits across species –

Variation in outcrossing rates in flowering plants was originally hypothesized to fit a bimodal distribution, due to the interaction between strength of inbreeding depression and

the automatic transmission advantage of selfing, in which populations will evolve towards stable endpoints of complete selfing or outcrossing (Lande and Schemske 1985). Early surveys supported the presence of a bimodal distribution (Schemske and Lande 1985, Barrett and Eckert 1990), but a more recent and extensive survey did not observe this pattern for animal-pollinated species (Vogler and Kalisz 2002). In the *Mimulus* taxa presented here, the distribution of outcrossing rates and related floral traits varied continuously, supporting the findings of the more recent survey. Taxa ranged, without substantial gaps, from highly inbred to moderately outcrossed, and no clusters of populations occurred at the highly outcrossed end of the mating system continuum (i.e., greater than 70 %). Continuous variation in mating system traits was observed in the Collinsieae, and the authors suggested two alternative hypotheses for the presence of intermediate forms: 1) selection is driving populations towards the mating system endpoints, but that phylogenetic lag or genetic constraints cause many populations to fall between the extremes or 2) correlational selection, acting on various floral traits, causes the fitness function to include continuous variation (Armbruster et al. 2002). As in the Collinsieae study, the observation of limited phylogenetic inertia supports the first hypothesis, but the extreme lability of mating system traits support the second hypothesis even more strongly.

Summary –

Mating systems in the *M. moschatus* alliance, and its sister clade, the *M. guttatus* complex, ranged from highly inbred to moderately outcrossed. Outcrossing rates were strongly correlated with a suite of floral traits; selfing taxa have small corollas with little anther-stigma separation and reduced pollen investment, while outcrossing taxa had large corollas with greater anther-stigma separation, and increased investment in pollen. Trait variation in this group fits the pattern of outcrossing taxa investing greater resources in male relative to female function. No tradeoff was detected between pollen number and volume. The selfing taxa appeared to be well adapted to reduced pollinator visitation, as autonomous seed set was quite high. Autonomous seed is apparently a complex function

of many traits, many of which are largely evolutinarily independent. Thus, the observed pattern of floral covariation best fits the notion of adaptation to specific mating system optima, ranging from nearly obligate selfing to moderately outcrossed. The high proportion of mixed mating taxa in this group adds support to the hypothesis that other genetic and ecological forces maintain intermediate mating systems.

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CHAPTER 3. THE EVOLUTION OF MATING SYSTEM AND INBREEDING DEPRESSION IN THE MIMULUS MOSCHATUS ALLIANCE

ABSTRACT

The relationship between inbreeding depression and mating system evolution was studied in a monophyletic clade of 14 populations (seven species) in the *M. moschatus* alliance, using a phylogenetic approach. Cumulative inbreeding depression ranged from –0.13 to 0.53, with an overall mean of 0.20. Outcrossing rates ranged from completely inbred to largely outcrossed ($t = 0.00$ to 0.69). When present, inbreeding depression occurred in both early and late life stages, and there was no evidence that the timing of inbreeding depression was earlier for outcrossing populations relative to selfing populations. Inbreeding depression was positively correlated with anther-stigma separation and outcrossing rates when treating all populations as independent ($R = 0.54$, $P < 0.05$ and $R = 0.53$, $P < 0.10$). Relationships were weaker and non-significant in the phylogenetically corrected analyses. Taxa inherited a substantial portion of mating system and, to a lesser degree, inbreeding depression values from their ancestors. Inbreeding depression appears to have evolved more rapidly than has outcrossing rates and floral morphology. I conclude that levels of self-fertilization and inbreeding depression in this clade are affected more by other factors than by each other.

INTRODUCTION

A recurrent evolutionary transition in flowering plants is the mating system shift from outcrossing to selfing lineages (Stebbins 1950, Grant 1981, Barrett, Harder, and Worley 1996, Takebayashi and Morrell 2001). A selfing mating system is recognized to have

two primary advantages: 1) reproductive assurance and 2) a twofold gene transmission advantage. The first advantage occurs when pollen or pollinators are scarce and individuals with the capacity for self-fertilization have a substantial reproductive benefit over obligate outcrossers (Baker 1955). The automatic gene transmission advantage occurs with the self-fertilization of all an individual's ovules and dispersal of pollen equal to that of obligate outcrossing individuals (Fisher 1941, Jain 1976). Additional hypotheses for the widespread presence of selfing mating systems include the maintenance of coadapted gene complexes at different loci (Stebbins 1957, Grant 1975, Solbrig, 1976) and a lower cost of producing selfed relative to outcrossed offspring (Schemske 1978, Waller 1979).

Theoretical models of mating system evolution predict that modifier alleles that promote greater self-fertilization will increase due to the transmission advantage (see review in Takebayashi and Morrell 2001). However, when inbreeding depression is high and the fitness of selfed offspring drops below half that of outcrossed offspring, the transmission advantage disappears and outcrossing is maintained. Inbreeding depression is a very widespread phenomenon in plants and can be quite severe (see review in Husband and Schemske 1996) and, as such, it is commonly invoked as one of the primary selective forces maintaining outcrossing (however, see Holsinger 1988).

Inbreeding depression is not a static force, but is believed to evolve in concert with selfing rates (Lande and Schemske 1985, Campbell 1986, Holsinger 1988, Johnston and Schoen 1996). Increased selfing exposes a greater proportion of deleterious, recessive (or partly recessive) alleles to selection. Thus, with increased selfing there should be a corresponding decline in inbreeding depression, at least when partial dominance is the primary cause of inbreeding depression (Lande and Schemske 1985). The role of inbreeding depression in mating system evolution is not altered when more realistic, coevolutionary models (i.e., selfing evolves incrementally and inbreeding depression is caused by deleterious alleles at unlinked loci) are applied (Lande and Schemske 1985, Charlesworth et al. 1990, Charlesworth et al. 1991).

Models of mating-system evolution suggest that, if inbreeding depression is due to deleterious recessive alleles, a positive association should quickly develop between "fitness"- associated alleles and alleles increasing self-fertilization rate (Holsinger 1988, Uyenoyama and Waller 1991a, b, Carr et al. 1997, Takebayashi and Delph 2000; however, see Schultz and Willis 1995). Strong selection should exist to combine the transmission advantage of selfing with non-deleterious alleles. In *Mimulus* sect. *Simiolus*, high selfing rates have evolved numerous times (Ritland 1989, Fenster and Ritland 1992, 1994a, Carr et al. 1997). Additionally, in this group, mating system is highly correlated with a suite of floral characters (Ritland and Ritland 1989, Dole 1992, Carr and Fenster 1994, Carr et al. 1997), many of which have been shown to be heritable (Carr and Fenster 1994, Robertson et al. 1994, Fenster and Ritland 1994, Fenster et al. 1995). For example, anther-stigma separation has been demonstrated to vary among species and among individuals within populations and is not only heritable, but is associated with selfing rates (Dole 1992, Carr and Fenster 1994). Interestingly, in *M. guttatus*, variation in inbreeding depression was not explained by anther-stigma separation or self-fertilization level (Carr et al. 1997). This calls into question the degree of linkage between fitness and selfing loci.

Broad associations between selfing rate (= 1 – outcrossing rate) and inbreeding depression are now recognized (see Husband and Schemske 1996) and an increasing number of experimental studies are helping to define the genetic basis of inbreeding depression in plants (Dudash and Carr 1998a, 1998b, Willis 1999a, 1999b, 1999c). However, many aspects of the interaction between inbreeding depression and mating system evolution remain ambiguous (Johnston and Schoen 1996). Further, while members of *Mimulus* sect. *Simiolus* (specifically *M. guttatus*) have been the subject of much mating system and inbreeding depression experimentation, other members of the genus have been largely ignored (however, see Meinke 1992, Bradshaw et al. 1995). In particular, understanding inbreeding depression, mating system, and their inter-correlation for species of conservation concern is of great importance (Karron 1989, Van Treuren et al. 1993, Kephart et al. 1999). Lastly, studies of the subject of the evolution of

mating system and correlated traits benefit from incorporating phylogenetic history to test explicitly evolutionary hypotheses (Armbruster 1993, Weller and Sakai 1999).

In the present study, I describe the magnitude and timing of inbreeding depression in 14 populations (seven species) in the *M. moschatus* alliance and address three main questions. Is inbreeding depression positively correlated with anther-stigma separation, outcrossing rate, pollen/ovule ratio, corolla size, and negatively correlated with autonomous seed set? Do taxa inherit a significant portion of both mating system traits and inbreeding depression alleles from their ancestors (i.e., is mating system and inbreeding depression as evolutionarily labile as generally assumed)? What is the sequence of evolutionary changes in mating system and inbreeding depression? Is there evidence that mating system (outcrossing rate) evolves prior to inbreeding depression rather than coevolving nearly simultaneously, and are high levels of inbreeding depression barriers to the evolution of selfing? Finally, are outcrossing rates more evolutionarily labile then morphological floral traits?

To address these hypotheses I have used the *M. moschatus* alliance (Scrophulariaceae) as a study system. By integrating data on inbreeding-depression levels, outcrossing rates, and floral morphology with phylogenetic information, I explore the historical relationship among these traits and the effect of ancestry. This study is part of a larger attempt to understand the links between evolution of floral morphology, fitness consequences of crossing distance, and implications for conservation biology.

METHODS

Study organisms—The *Mimulus moschatus* alliance is a clade of 12 moderate to small-flowered, viscidly pubescent species, with a broad range of outcrossing rates ($t = 0.00 - 0.69$; see Chapter 2). All members of the alliance are self-compatible, and most will autonomously self-pollinate in the absence of insect visitation. Eight of the twelve species are extremely geographically restricted, prompting attention from conservation organizations (Oregon Natural Heritage Program 2001). Additionally, most of the

species occur in small, isolated populations. Three species are very widespread. Locations of study populations are represented in Figure 3-1.

I estimated inbreeding depression levels and mating system traits in two populations each of a subset of seven species in the *Mimulus moschatus* alliance. All populations were located over two kilometers apart, and most were over ten. Distances of these magnitudes have been large enough to generate fitness differences in between-populations crosses of *Chamaecrista fasciculata* (Fenster and Galloway 2000). Additionally, most plant populations have quite restricted neighborhood sizes and high population subdivision (Levin 1981, Fenster 1991, Karron et al. 1995), so that crosses between plants separated by only tens of meters should result in mixing of largely disparate gene pools. The species included in the analysis fall into three clades: the "Snake River clade" (*M. ampliatus*, *M. hymenophyllus*, and *M. patulus*), the "Columbia River clade" (*M. washingtonensis* and *M. jungermannioides*), and the "Sierra Nevada clade" (*M. dudleyi* and *M. floribundus*) (Whittall, et al. in press). Figure 3-2 shows the strict-consensus composite tree (i.e., supertree, sensu Bininda-Emonds and Sanderson 2001), based on two chloroplast, two nuclear, and a mating system-independent morphological data sets (see Chapter 1). Supertree analysis does not include information about branch lengths, so all branch lengths were assigned equal lengths, except population-level branches, which were given lengths half those of others. This decision was made because branch lengths from ITS and chloroplast species trees tended to be quite similar (Whittall et al. unpublished manuscript, Beardsley unpublished manuscript), and morphologically the populations within species showed some differentiation, but not worthy of species-level branch lengths (see Chapter 2).

Inbreeding depression estimation –

I grew individuals from field-collected seed of 60-80 randomly selected, plants per population. Seeds from the Snake River clade and *M. washingtonensis* were cold-

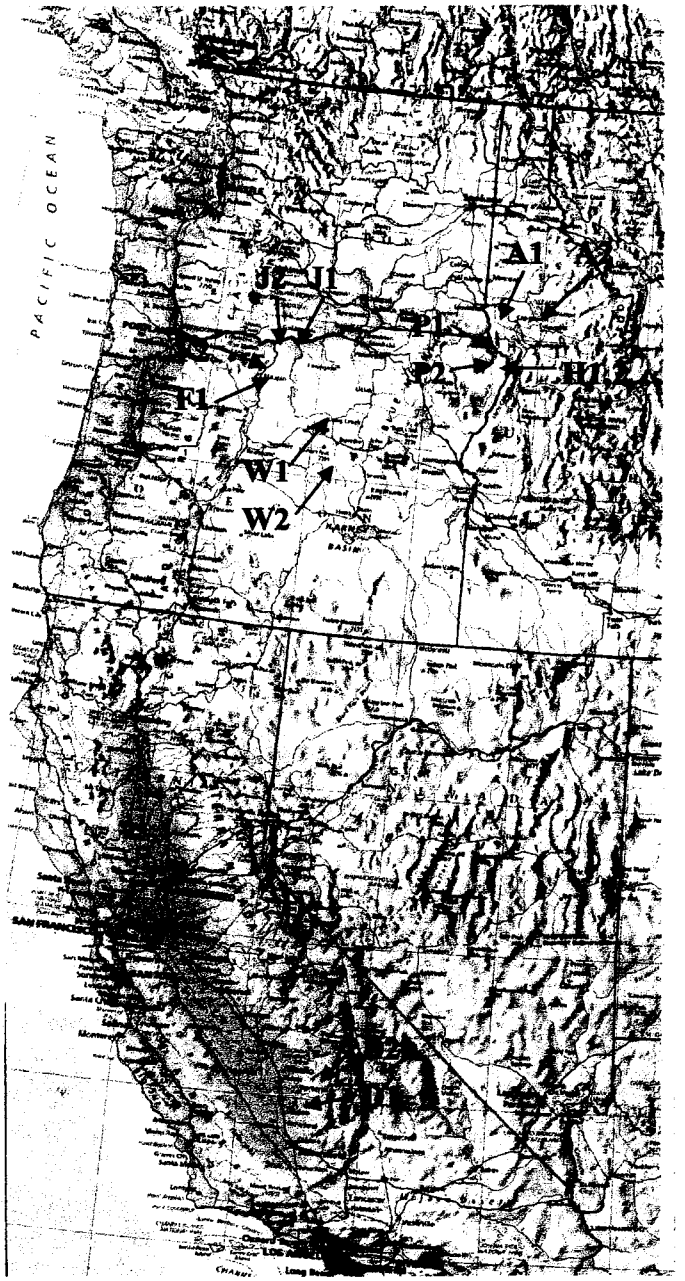


FIGURE 3-1. Distribution of source populations for estimating inbreeding depression levels and mating system. A1 and A2 = *M. ampliatus* populations 1 and 2, D1 and D2 = *M. dudleyi* populations 1 and 2, F1 and F2 = *M. floribundus* populations 1 and 2, H1 and H2 = *M. hymenophyllus* populations 1 and 2, J1 and J2 = *M. jungermannioides* populations 1 and 2, P1 and P2 = *M. patulus* populations 1 and 2, W1 and W1 = *M. washingtonensis* populations 1 and 2.

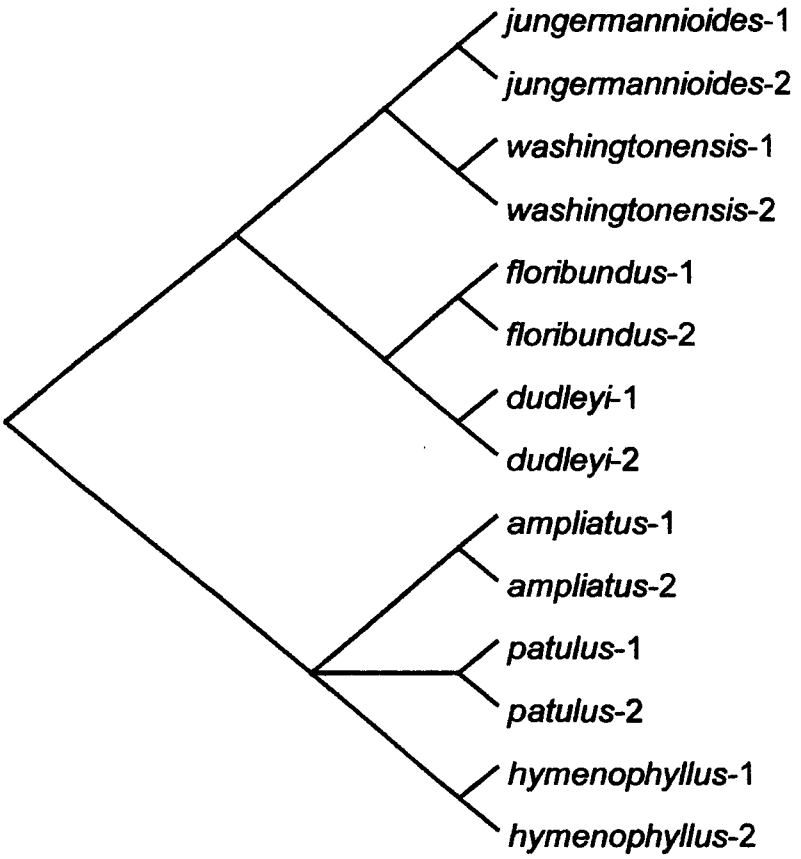


FIGURE 3-2. The strict-consensus supertree for the *Mimulus moschatus* alliance. Four species in the *Mimulus moschatus* alliance lacking inbreeding-depression estimates were pruned from the tree (see Chapter 1 for a complete tree). Branch lengths were assumed equal, except terminal branches to populations, which were treated as half the length of all others.

stratified in the dark at 4°C for approximately 70 d, prior to germination. All seeds were germinated on moist paper in small plastic zip-lock bags under 16 h day length and 22°C in the University of Trondheim, Norway, Botany Department greenhouse. Upon germination, a single randomly selected seedling per family was transferred to its own 730 cm^3 pot, with standard potting mix ("Elg P-Jord:" 50 % humus, 25 % sand, 25 % perlite). All plants were watered from below as needed; however, no fertilizer was added during the study. All species were grown in the greenhouse from November 1998 to February 1999 under the same conditions.

Only one individual per family was used to generate selfed and outcrossed offspring. All crosses were reciprocal, such that each individual served as both a pollen donor and pollen recipient to each crossing partner (see Lynch 1988, Lynch and Walsh 1998). Because few families were represented, multiple crosses per individual were made in an incomplete diallel, for both populations of *M ampliatus*. Normally, these species produce two flowers per node, and self and outcross pollinations were paired on these nodes to reduce positional effects (Latta and Ritland 1994, Carr and Dudash 1996). In cases where only one flower per node was produced, or if the two flowers were too developmentally disparate, flowers were chosen haphazardly. All experimental flowers were emasculated prior to anthesis. The two small-flowered species, *M. patulus* and *M. floribundus*, were emasculated while in bud, about one to two days prior to opening, because anther dehiscence occurs just prior to the flower opening. All other species were emasculated either one day prior to, or the day of, floral opening. Ten to 15 control flowers per population were emasculated to confirm that no pollen contamination occurs. Flowers were pollinated two hours to one day following emasculation to allow the stigmas to become receptive and the stigmatic lobes to reopen after being touched. *Mimulus* stigmas are thigmatropically sensitive and will reopen if insufficient pollen is deposited (Newcombe 1922, Meinke 1992, Fetscher and Kohn 1999). Hand pollinations were performed by brushing a single mature anther against the stigma until the stigmatic lobes closed around the anther. This resulted in complete covering of the stigmatic surface with ca. > 20 times the number of pollen grains to ovules. For the four

populations in the Sierra Nevada clade, 43 to 48 plants per population were crossed, and in the four populations in the Columbia River clade, 57 to 62 plants per population were crossed. The six populations in the Snake River clade had poor germination and as a result between six and 18 maternal plants per population were crossed.

Fruits were collected three to five weeks following pollination, as they became dry, but before dehiscence. Ten or 20 seeds per capsule (or less if fewer seeds were set) were germinated in similar conditions to their parents. Progeny from each mature capsule were grown in the Oregon State University, Botany Department, greenhouse from June 1999 to September 2000 in three blocks under conditions similar to their parents. Five germinated seeds were randomly selected and planted in pots (ca. 730 cm^3) with a standard potting mix ("Witham Farms Standard Mix:" 50 % humus, 25 % sand, 25 % perlite). A total of 2160 individuals were planted. After 60 d, the percentage of germinated seeds was scored.

Progeny were grown with 16 h light at 22^0C and 8 h dark at 15^0C. They were watered from below as needed, but not fertilized. Six of the seven species are annuals and they were allowed to grow until they naturally senesced. *Mimulus jungermannioides* is a perennial, and it was not watered after the last of the other annuals senesced, i.e., after ca. 3.5 mo. In addition to number of seeds per maternal fruit and germination percent, I measured offspring survival and total number of flowers per plant. These measures together seem to be a good estimate of fitness over a single generation (Latta and Ritland 1994, Kephart et al. 1999). They include components of multiple life-history stages: embryo and seedling fitness (seed set and germination percent), and "mid-life" and lifetime fecundity (survival and flower number). A composite measure of fitness was defined as seed number times germination percent times survival times flower number.

Analysis of Data –

Fitness differences between outcrossed and selfed progeny were compared at each life history stage using the method proposed by Lynch (1988). Each pair of parents were both selfed and reciprocally outcrossed. Because the two parents contribute equal

numbers of genes to outcrossed and selfed progeny, variance from maternal environment effects and parent sampling do not contribute to the variance of the mean difference between treatments (see Lynch and Walsh 1998). Additionally, we measured relative performance (RP_{ws}) for each experimental unit (a self-outcross pair) following the method proposed by Ågren and Schemske (1993). In this measurement $RP_{ws} = (W_{ow} - W_s)$/maximum (W_{ow}, W_s), where W_{ow} is the cumulative fitness for the within-population cross and W_s is the cumulative fitness of a selfed cross. Positive RP_{ws} values indicate that the performance of outcrossed individuals exceeds that of selfed individuals (inbreeding depression). Negative values indicate that selfed individuals outperform outcrossed individuals. +1 and –1 bound this measure. For positive values, RP_{ws} is identical to the traditional measure of inbreeding depression: $\delta = 1$ - (self/outcross). However, the traditional measure is bounded by +1 and -∞ and if negative values occur the population mean is weighted towards within-population outbreeding depression (Carr et al. 1997).

I analyzed each population separately using SPSS Base 8.0. Differences at each fitness stage and cumulative fitness (RP_{ws}) were analyzed with one-sample *t*-tests. Population-wise Bonferroni correction was used to maintain the probability of committing type I error at 0.05. Differences among species in levels of inbreeding depression were analyzed with nested ANOVA (populations within species).

To test whether there is a positive association between mating-system characters and inbreeding depression and what effect evolutionary history has had on those characters, correlation analysis was employed in two ways: on phylogenetically "naïve" and phylogenetically corrected data. The results of the phylogenetically naïve and corrected data were compared to interpret the extent of phylogenetic effect and the nature of the phylogenetic signal (see Armbruster et al. 2002). To achieve a phylogenetically corrected analysis, Felsenstein's (1985) independent contrasts were employed using the computer program CAIC (Purvis and Rambaut 1995). Additionally, Phylogenetic Mixed Model analysis was used to estimate the "phylogenetic heritability" or relative importance of phylogeny in explaining the phenotypic variation (Lynch 1991) in Martins'

(2001) computer program, COMPARE version 4.4. In this model each observation ($\check{z}_{ci}$) can be expressed as the linear function

$$\check{z}_{ci} = \mu_c + a_{ci} + e_{ci}$$

where μ_c is the grand mean of the cth character over the entire phylogeny, a_{ci} is the heritable additive value (the phylogenetic effect of Cheverud et al. 1985) of the character in the ith taxon (measured as the deviation from the grand mean), and e_{ci} is the residual deviation from the predicted value, which can be due to non-additive genetic effects, environmental effects, and sampling error (Lynch 1991). Thus, the additive values here are analogous to breeding values in quantitative genetics (Falconer 1981), with the quantity $\mu_c + a_{ci}$ interpreted as the phylogenetically heritable component of the mean phenotype of the ith taxon. Lynch (1991) then derives phylogenetic heritability as

$$H^2_P = \sigma^2_a / \sigma^2_T$$

where σ^2_a is the variation due to additive effects and σ^2_T is the total variation. Differences in H^2_P among characters may provide information on to what extent various traits display phylogenetic inertia (Lynch 1991). When phylogenetic heritability values are high (near 1.0) the degree of resemblance among relatives is high (i.e., high degree of phylogenetic inertia); when values are low (near zero) a high degree of randomness in trait expression is present throughout the phylogeny or trait responses are to "local" selection.

To examine the degree of coevolution of mating system and inbreeding depression and to test whether high levels of inbreeding depression represent a barrier to the evolution of a selfing-mating system, I employed Martins and Hansen's (1997) Generalized Least Squares approach to estimate ancestral states in the program, COMPARE version 4.4 (Martins 2001). Transitions in estimated ancestral states from one ancestral node to the next were then compared across the phylogeny. Last, to test whether floral characters are more evolutionarily constrained than outcrossing rates, Martins and Hansen's (1997) Generalized Least Squares approach was taken to estimate the α-parameter, or the strength of evolutionary constraint. The value of $\ln(2)/\alpha$ can be translated as the half time necessary for the trait of interest to meet a new adaptive

optimum in response to a change in the selective environment (Hansen 1997). This gives a rough time scale at which adaptation occurs, with values near zero indicating the optimum will never be reached, and values near infinity meaning that adaptation is instantaneous (Hansen 1997).

RESULTS

Inbreeding depression values (RP_{WS}) varied significantly among species (Table 3-1), with mean population-level values ranging from –0.13 to +0.53 (Table 3-2). The overall mean inbreeding depression level for the *M. moschatus* alliance was 0.20. Thus, selfing reduced fitness by about 20 % on average for these populations. Three populations had higher mean cumulative fitness of selfed relative to outcrossed progeny (Table 3-2); however, the values were not significantly different from zero (see below). Two of these populations habitually self ($t < 0.10$).

Both populations of the extremely geographically restricted, mixed-mating species, *M. dudleyi* displayed significant cumulative inbreeding depression. The most outcrossing species in the alliance, *M. washingtonensis*, had one population with the highest degree of cumulative inbreeding depression (0.53) while inbreeding depression in the other population was not significantly different from zero. Both of these populations had similar corolla sizes, anther-stigma separation, and outcrossing rates (see Chapter 2). The more selfing populations did not display significant levels of cumulative inbreeding depression. However for the more selfing populations, a particular life-stage sometimes showed evidence of inbreeding depression (Table 3-2).

Overall, there did not seem to be a pattern in the timing of expression in inbreeding depression in this group. The expression of inbreeding depression at one life-stage appeared to be independent of expressions at other stages. Inbreeding depression was expressed at all life-stages, but with most populations expressing it at the germination and survival stages rather than early (seed set) or late (flower production) in life (Table 3-2).

TABLE 3-1. Nested ANOVA of inbreeding depression in the *Mimulus moschatus* alliance, measured as relative performance of outcrossed to selfed progeny (RP_{WS}).

Source	df	MS	F	P
Species	7	2.996	5.211	0.022
Population within species	7	0.575	1.560	0.147
Error	276	0.368		

TABLE 3-2. Mean inbreeding depression for four life-history stages, plus cumulative fitness in the *Mimulus moschatus* alliance, measured as relative performance of outcrossed to selfed progeny for seeds produced/fruit, percent germination and survival, total number of flowers/plant, plus cumulative fitness. Outcrossing rates (t) are presented for all populations, except *M. patulus*-1, which was monomorphic for all allozyme loci. Sample sizes (reciprocal pairs of parents) are give below population names. Standard errors are in parentheses. † = P < 0.10, * = P < 0.05, ** = P < 0.01.

Species (population)	Seed set	Germ. %	Survival %	Flower number	Cum. fitness	t
M. ampliatus-1	-0.026	0.334*	-0.084	0.148	0.241	0.00
15	(0.056)	(0.092)	(0.180)	(0.141)	(0.204)	
M. ampliatus-2	0.116†	0.094	0.018	0.225	0.181	0.06
28	(0.645)	(0.123)	(0.118)	(0.140)	(0.113)	
M. dudleyi-1	0.102	0.314**	0.236*	0.116	0.468**	0.41
26	(0.081)	(0.083)	(0.080)	(0.136)	(0.116)	
M. dudleyi-2	0.202	0.267**	0.177	-0.054	0.459**	0.47
24	(0.087)	(0.073)	(0.089)	(0.113)	(0.115)	
M. floribundus-1	0.023	-0.054	0.003	0.004	-0.109	0.00
20	(0.115)	(0.084)	(0.079)	(0.134)	(0.172)	
M. floribundus-2	-0.017	0.095	0.062	0.002	0.145	0.01
22	(0.099)	(0.068)	(0.066)	(0.068)	(0.124)	
M. hymenophyllus-1	-0.082	-0.141	-0.187	0.310	-0.129	0.44
11	(0.137)	(0.139)	(0.178)	(0.151)	(0.229)	
M. hymenophyllus-2	0.126	-0.299	-0.101	0.124	0.347	0.40
11	(0.109)	(0.213)	(0.213)	(0.188)	(0.237)	
M. jungermannioides-1	-0.034	0.033	0.076	0.060	0.042	0.30
30	(0.053)	(0.035)	(0.061)	(0.062)	(0.096)	
M. jungermannioides-2	0.057	0.075	0.130*	-0.004	0.213†	0.45
30	(0.065)	(0.059)	(0.056)	(0.079)	(0.106)	
M. patulus-1	0.095	-0.159	0.433**	0.165	0.218	-
13	(0.167)	(0.170)	(0.130)	(0.271)	(0.239)	
M. patulus-2	-0.109	0.157	-0.084	0.169	-0.027	0.09
11	(0.118)	(0.133)	(0.130)	(0.151)	(0.215)	
M. washingtonensis-1	0.063	0.086	0.038	0.104	0.172	0.60
31	(0.066)	(0.070)	(0.086)	(0.105)	(0.121)	
M. washingtonensis-2	0.258*	0.122*	0.088	0.248*	0.529**	0.69
30	(0.079)	(0.041)	(0.066)	(0.076)	(0.073)	

Association of inbreeding depression with mating system –

The correlations between mating-system traits and inbreeding depression were weak for this species alliance, in both naïve and phylogenetically corrected analyses (Table 3-3, Fig. 3-3). All correlations were in the expected direction, i.e., positive between inbreeding depression and anther-stigma distance, outcrossing rates, corolla size, and a negative correlation with autonomous seed set. Anther-stigma separation was the only trait that was significantly correlated with degree of inbreeding depression in the phylogenetically naïve analysis ($R = 0.538$, $P < 0.05$). Outcrossing rate was marginally significant ($R = 0.533$, $P < 0.10$, $R = 0.470$, $P < 0.10$, t and pollen number, respectively). Relationships in the phylogenetically corrected correlations were all weaker than in the naïve analysis and none was significant (Table 3-10).

Phylogenetic effect –

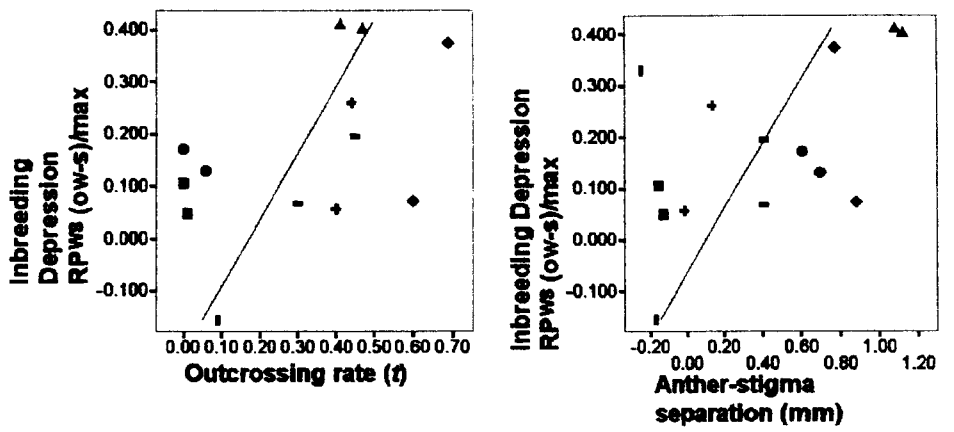
Phylogenetic relationship was important in explaining the magnitude of mating system, while phylogenetic relationship was less important in explaining levels of inbreeding depression. Phylogenetic heritabilities (Lynch 1991) of mating-system traits (outcrossing rate, anther-stigma separation, and corolla size) all approached 1.00. This indicates that the taxa's phenotypic information provided a large amount of information about each other (Lynch 1991). Inbreeding depression on the other hand, had a much lower phylogenetic heritability ($H^2_P = 0.363$), indicating that phylogenetic history was of much less importance to this trait relative to mating-system traits. A low H^2_P value implies low phylogenetic inertia, where a high degree of "randomness" in the trait's magnitude is expressed throughout the phylogeny. Within-species contrasts were of lesser magnitude than higher-taxonomic level contrasts for outcrossing rates ($t = 3.92$, $df = 9$, $P < 0.01$; Fig. 3-4.). Anther-stigma separation showed a tendency to follow this pattern ($t = 1.62$, $df = 9$, $P = 0.14$). However, this was clearly not the case for degree of inbreeding depression ($t = 0.043$, $df = 9$, $P > 0.50$).

TABLE 3-3. Correlations between inbreeding depression (cumulative performance of outcross-within population progeny relative to self-progeny) and mating system indicators among 14 populations (seven species) in the *M. moschatus* alliance. Phylogenetically naïve correlations are presented in the second column; phylogenetically corrected correlations (Felsenstein's Independent Contrasts) are presented in the third column. The number of phylogenetic contrasts $n = 11$. P values are represented after Pearson correlation coefficients. * = $P < 0.05$, † = $P < 0.10$; two tailed test. None of the phylogenetically corrected correlations are significant. After overall table-wise Bonferroni adjustment, naïve correlations are also not significant.

	Naïve	Phylogenetically corrected
Outcrossing rate (t)	0.533†	0.336
Seed set	-0.237	-0.067
Corolla height	0.418	0.394
Anther-stigma separation	0.538*	0.427

A.

B.



C.

D.

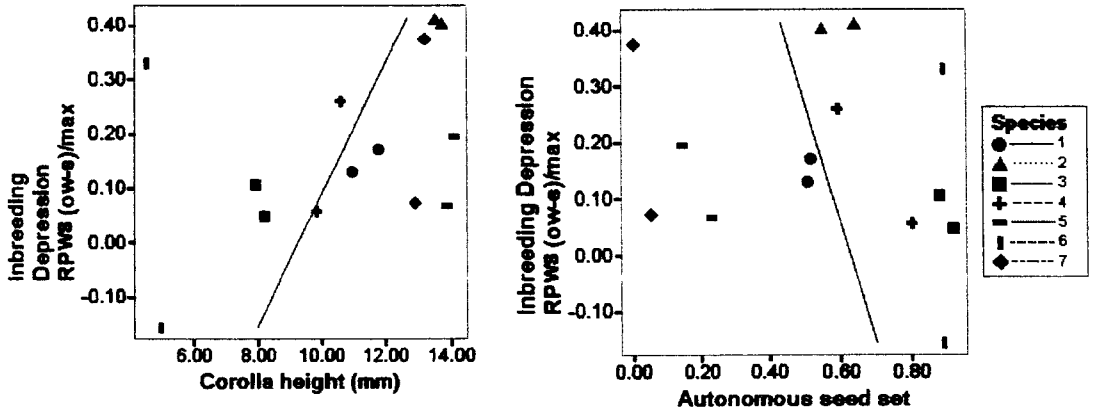


FIGURE 3-3. Relationship between mean inbreeding depression and mating system traits for 14 populations (treated as independent). Inbreeding depression is measured as RP_{WS}, i.e., relative performance of (outcross-self)/max. A) shows the relationship between RP_{WS} and outcrossing rate. B) shows the relationship between RP_{WS} and anther-stigma separation. C) shows the relationship between RP_{WS} and corolla height. D) shows the relationship between autonomous seed set (seeds/ovule). Species 1 = *M. ampliatus*, 2 = *M. dudleyi*, 3 = *M. floribundus*, 4 = *M. hymenophyllus*, 5 = *M. patulus*, 6 = *M. washingtonensis*.

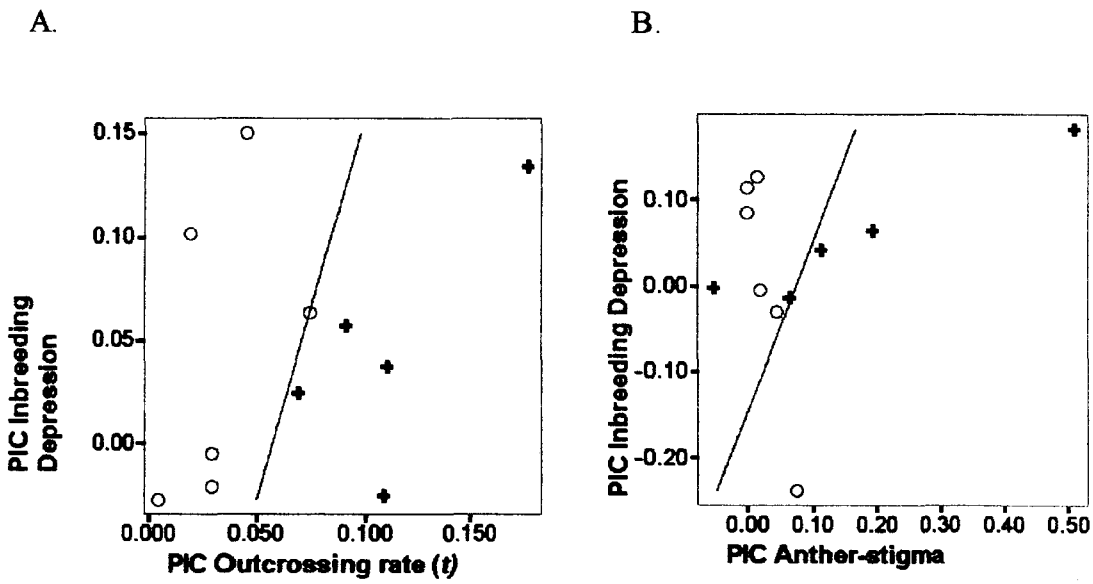


FIGURE 3-4. Phylogenetically independent contrasts between inbreeding depression and mating system in the *M. moschatus* alliance. A) shows the relationship between independent contrasts of outcrossing rate and inbreeding depression measured as RP_{WS}, i.e., relative performance of (outcross-self)/max and B) shows the relationship between independent contrasts inbreeding depression and anther-stigma separation. Between population contrasts are indicated as circles, among species and higher level contrasts are indicated as plusses. No differences in inbreeding depression or anther-stigma separation contrasts are present between populations of the same species relative to higher taxonomic levels (RP_{WS}: $t = 0.043$, $df = 9$, $P > 0.50$; anther-stigma: $t = 1.62$, $df = 9$, P 0.14). However, contrasts in outcrossing rates are significantly greater among higher levels relative to those between populations of the same species ($t = 3.92$, $df = 9$, $P <$ 0.01).

Evolutionary sequence of mating system- and inbreeding depression-magnitudes – Mating system and inbreeding depression seem to evolve in concert, based on ancestral state reconstruction (Fig. 3-5). However, by comparing successive evolutionary nodes, there were cases where inbreeding depression remained constant or increased slightly, while outcrossing rates dropped. There were also cases where inbreeding depression decreased, while outcrossing rates increased. There was no consistent pattern of the evolutionary sequence of the two traits and thus, there is no evidence that mating system evolves prior to inbreeding depression rather than simultaneously. The estimated ancestral states of inbreeding depression was never greater than 50 % and thus, did not appear to represent a constraint on the evolution of selfing in this group.

There was an indication that outcrossing rates were more evolvable than floral traits. Figure 3-6 shows the estimated ancestral states of outcrossing rates and anther-stigma separation. The maximum likelihood estimates of the α-parameter (i.e., the strength of evolutionary constraint) for anther-stigma separation and corolla height were 1.50 and 0.55, respectively. The maximum likelihood estimate of α for outcrossing rate was 8.00. The value of $\ln(2)/\alpha$ can be translated as the half time necessary for the trait of interest to meet a new adaptive optimum in response to a change in the selective environment (Hansen 1997), giving a rough time scale at which adaptation occurs. This suggested that the half time to respond to selection for anther-stigma separation and corolla height is 46,000 and 1,260,000 years, respectively. The half to respond to selection for outcrossing rates is only 8,700 years. Thus, these morphological traits have evolved from 5 to 15 times more slowly than do outcrossing rates.

DISCUSSION

Description of inbreeding depression in the species (populations) – Inbreeding depression and mating system were found to vary widely among populations and species in the *M. moschatus* alliance. Outcrossing rates ranged from complete

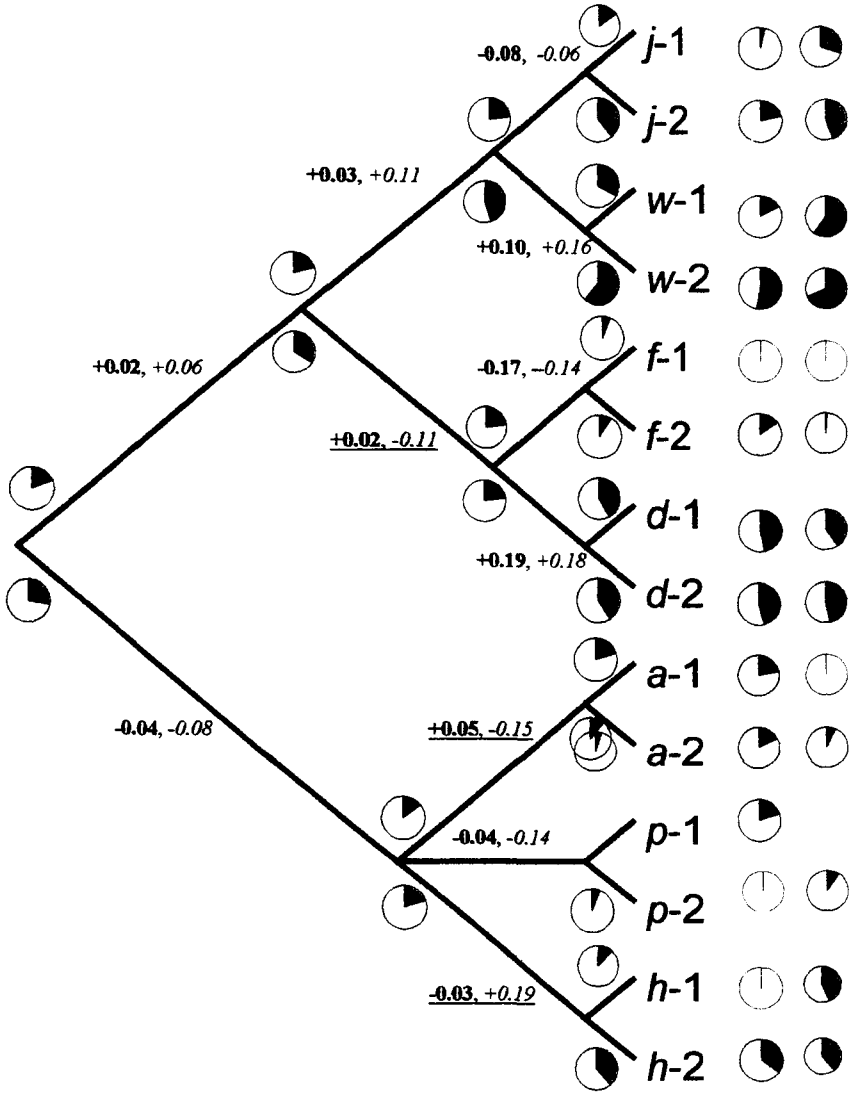


FIGURE 3-5. Estimated ancestral inbreeding depression and outcrossing rates mapped on a composite phylogeny of selected members of the *Mimulus moschatus* alliance. The phylogeny is based on a composite tree of two chloroplast, two nuclear, and one mating system-independent morphological trees (Carlson et al. unpublished manuscript). Estimated ancestral inbreeding depression (black) and outcrossing rates (dark gray) are shown. The numbers indicate the change in trait values between nodes. Bold = inbreeding depression, italics = outcrossing rates. Ancestral state-estimation is based on Generalized Least Squares (Martins 2002).

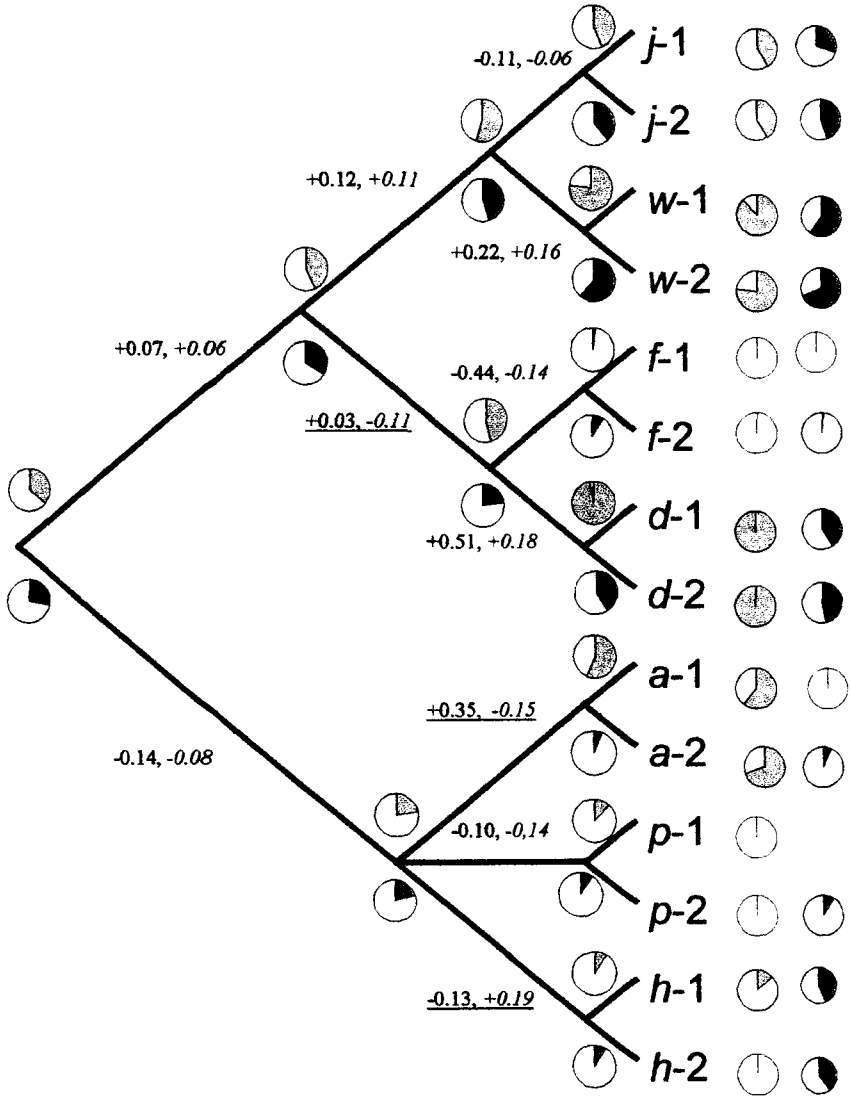


FIGURE 3-6. Estimated ancestral outcrossing rates and anther-stigma separation mapped on a composite phylogeny of selected members of the *Mimulus moschatus* alliance. The phylogeny is based on a composite tree of two chloroplast, two nuclear, and one mating system-unbiased morphological trees (Carlson et al. unpublished manuscript), showing the estimated ancestral level of outcrossing rates (dark gray) and anther-stigma separation (light gray). The numbers indicate the change in trait values between nodes. Italics = outcrossing rates, and normal = anther-stigma separation. Underlined numbers indicate estimated transitions of the two traits in opposite directions. Ancestral state-estimation is based on Generalized Least Squares (Martins 2002).

inbreeding to around 70 % outcrossing, and cumulative inbreeding-depression values ranged from -13 % to +53 %. However, most populations did not have inbreeding depression levels significantly different from zero. Three of 14 populations did have significant cumulative fitness declines of between 46 and 53 % upon selfing. Relative to most of the other populations, these three populations had high outcrossing rates (t = 0.41-0.69), large anther-stigma separation, and large corollas. However, not all outcrossing populations experienced high levels of inbreeding depression. One population of the most outcrossing species in the species alliance (*M. washingtonesis*) had an inbreeding depression level of only 17 %. Additionally, a few populations experienced a minor (although non-significant) fitness advantage of selfing relative to outcrossing. These populations generally had low outcrossing rates. Many populations that did not display significant cumulative inbreeding depression experienced substantial levels at one or two of the four life stages, but not at others.

There was no detectible trend for more selfing species to express inbreeding depression later, rather than earlier in life than outcrossers. In a review of numerous studies, Husband and Schemske (1996) found that selfing species expressed more inbreeding depression late, during growth and reproduction, while inbreeding depression is commonly expressed at both early and late stages for outcrossing species. Templeton and Read (1983) suggest that early-acting inbreeding depression may have a larger component of lethal mutations, while late-acting inbreeding depression is composed of mildly deleterious mutations that are difficult to purge. Thus, because deleterious recessive alleles of large effect are easy to purge relative to polygenic mildly deleterious alleles (Lande and Schemske 1985, Charlesworth and Charlesworth 1987), early-acting inbreeding depression could quickly be lost in selfing species (Husband and Schemske 1996).

The timing of inbreeding-depression expression at any one life-stage appeared independent of expression at any other stages, a pattern also observed by Husband and Schemske (1996). The observation of little correlation in inbreeding depression across stages indicates that genes causing inbreeding depression are somewhat independent

(Husband and Schemske 1996). Thus, there was no evidence here that inbreeding depression in selfing populations is more associated with mildly deleterious alleles relative to more seriously deleterious or lethal alleles in outcrossing populations.

In general, sample sizes in this study did not appear to be large enough to detect small levels of inbreeding depression. Confidence intervals tended to be large (mean 95 % CI for all populations = ±0.33), casting doubt on the precision of estimated means, further used in correlation analyses. However, 95 % CI for most of the populations with non-significant cumulative inbreeding depression were below the biologically meaningful value of 0.50 (see Lande and Schemske 1985, Charlesworth and Charlesworth 1987). Thus, while the strength and significance of some correlations might be affected by the imprecision, I believe the general conclusions should not be affected.

Association of mating system and inbreeding depression –

This study investigated among-population variation in both mating system and inbreeding depression. I did not find strong support for an association between selfing enhancers and low inbreeding depression, as are predicted to exist when inbreeding depression is caused by partially recessive deleterious alleles (Uyenoyama and Waller 1991a, Takebayashi and Delph 2000). For the *M. moschatus* alliance populations, the correlation between mating system traits and inbreeding depression was generally weak in the both the naïve and phylogenetically corrected analysis. However, all correlations were in the expected direction. Correlations were positive between inbreeding depression and outcrossing rates, anther-stigma separation, pollen/ovule ratios, corolla size, and correlations were negative with autonomous seed set. Anther-stigma separation was the only trait that was significantly correlated with degree of inbreeding depression in the naïve analysis. Relationships in the phylogenetically corrected correlations were all weaker than in the naïve analysis and none were significant.

A positive association was found between inbreeding depression and anther-stigma separation within a highly morphologically variable population of *Gilia achilleifolia* (Takebayashi and Delph 2000), supporting the theory of an association

developing between selfing enhancers and fitness loci. However, in less morphologically variable populations of *M. guttatus*, Carr et al. (1997) did not find such evidence. In addition to a lack of substantial variation in mating system and inbreeding traits, a high degree of pollen discounting may slow down or eliminate the genetic association by reducing gene flow from selfers to outcrossers (Takebayashi and Delph 2000). Further, it may be difficult to detect genetic associations depending on their genetic background (Schultz and Willis 1995). In a multilocus simulation study, Schultz and Willis (1995) found that when inbreeding depression is due to a few highly deleterious mutations, a positive association develops with selfing enhancers, but when inbreeding depression is due to many mutations of individually small effect, a negative association was actually observed. Inbreeding depression in *M. guttatus* (sister to the *M. moschatus* alliance, Beardsley unpubl.) appears to be due primarily to many alleles of individually small effect (Willis 1999c). Thus, assuming a similar genetic basis in the *M. moschatus* alliance, it is not surprising that this study did not detect a significant correlation between mating system and inbreeding depression.

Phylogenetic effects –

Mating system evolutionary studies are now largely being directed at the within-population level (e.g., Dudash 1990, Carr et al. 1997, Takebayashi and Delph 2000). However with recent methodological advances, a broader and historical perspective can be robustly employed to test hypothetical patterns (Hansen 1997), largely derived from micro-evolutionary studies. Studies that use a phylogenetically related group of species can estimate the historical sequence of character transformations through time (Schulter et al. 1997 – check reference), and they can estimate the statistical effect of ancestry (Lynch 1991). The effect of ancestry can be subsequently removed to allow unbiased statistical comparisons, and the magnitude of ancestry effect can also be of primary interest.

In the *M. moschatus* alliance, there was a disparity between the effect of phylogenetic history (ancestry) on mating system-traits relative to inbreeding depression.

Phylogenetic history was more important in explaining the magnitude of mating system-traits than it was explaining inbreeding depression. Phylogenetic heritabilities (see Lynch 1991) of outcrossing rates, anther-stigma separation, pollen number, pollen-ovule ratio, and corolla size all approached 1.00, phylogenetic heritabilities of inbreeding depression were around 0.36. The relatively low heritability of inbreeding depression indicates that phylogenetic inertia is low, and a high degree of "randomness" in the character's magnitude is expressed throughout the phylogeny. The disparity in evolvabilities of mating system and inbreeding depression is also expressed in differences in magnitude of within-species independent contrasts to higher-taxonomic level contrasts.

This result suggests that mating-system modifiers and inbreeding depression alleles evolve differently, and it indicates a weaker association than has commonly been invoked (Uyenoyama and Waller 1991a, b, Takebayashi and Delph 2000; however, see Schultz and Willis 1995). It appears that fitness-associated alleles accumulate and are lost at a faster rate than are outcrossing rates or associated floral traits. This is surprising because population-level outcrossing rates incorporate the effect of the loss of alleles through drift (Latta and Ritland 1994). Additionally, outcrossing rates likely fluctuate through ecological time due to variation in pollinator service (Delph 1990, Kephart et al 1999.) Further, if inbreeding depression in this group is largely due to many alleles of small effect, they should be difficult to purge, and thus a substantial proportion should be inherited from one population or species to the next. It may be that random deleterious mutations of large effect are relatively common and since some amount of selfing occurs in all these population, those mutations of large effect would be lost relatively quickly (Lande and Schemske 1985). This scenario would result in a somewhat random pattern of inbreeding depression increasing and decreasing, with a minor effect of mating system, much like what was observed in this study.

Evolutionary sequence of mating system- and inbreeding depression-magnitudes – In general, mating system and inbreeding depression seem to largely evolve in concert, based on ancestral state estimation. However, by comparing successive evolutionary

nodes, there were cases where estimated inbreeding depression remained constant or increased slightly, while estimated outcrossing rates dropped. There were also cases where inbreeding depression decreased, while outcrossing rates increased. If these patterns are not the result of estimation error, they suggest a de-coupling of evolutionary trends of these two traits. It should be stressed however, that ancestral state estimation suffers from lack of certainty, as ancestral conditions are essentially means of extant taxa (Schulter et al. 1997, Hansen pers. comm.); further, not all members of the clade are included in the study. Regardless, these data cast doubt on a purely one-to-one coevolution of mating system and inbreeding depression. I propose that random mutation events may be more important than the purging effect associated with degree of selfing in influencing inbreeding depression levels.

Additionally, there was no consistent pattern of the evolutionary sequence of the two traits. In some instances the magnitude of outcrossing rates (or anther-stigma separation) appeared to evolve prior to inbreeding depression and in other cases the opposite occurred. Thus, there is no evidence that mating system evolves prior to inbreeding depression rather than coevolving (see Johnston and Schoen 1996). Because inbreeding depression levels of extant taxa was generally fairly low (20%) and only one population had levels above 50%, estimation of ancestral states did not reveal ancestors with inbreeding depression levels high enough to promote the evolution of outcrossing from selfing (see Lande and Schemske 1985, Holsinger 1988). Further, if estimated ancestral outcrossing rates are accepted, they are low enough (< 0.50) not to select for an outcrossing mating system (under the basic theoretical models; Lande and Schemske 1985, Charlesworth and Charlesworth 1987). Therefore, the observed shift from more selfing ancestors to the largely outcrossing species, *M. dudleyi* and *M. washingtonensis*, requires an alternative explaination.

Evolution of outcrossing rates and floral morphology –

Population-level outcrossing rates appeared to be more evolvable than morphological traits as expected. The maximum likelihood estimates of the strength of evolutionary

constraint for anther-stigma separation and corolla height were 5 and 15 times greater, respectively, than outcrossing rates. The evolutionary constraint can be translated to the half time necessary for the trait of interest to meet a new adaptive optimum in response to a change in the selective environment (Hansen 1997). This suggests that the half time to respond to selection for anther-stigma separation and corolla height is 46,000 and 1,260,000 years, respectively, the half time to respond to selection for outcrossing rates is only 8,700 years. Thus, outcrossing rates are likely influenced by a number of ecological parameters independent of morphology. The two primary factors in this case are likely fluctuations in pollinator abundance and changes in population size. This result supports the need to investigate actual outcrossing rates rather than morphological correlates in relation to inbreeding depression.

Conservation implications –

Most populations in the *M. moschatus* alliance did not experience severe levels of inbreeding depression. However, a few rare species did. Both populations of the severely geographically restricted, *M. dudleyi*, had inbreeding depression levels of around 0.45 and one population of the rare, *M. washingtonensis* had inbreeding depression levels of over 0.50. Since high inbreeding depression is a crucial predictor of population extinction (Newman and Pilson 1997, Saccheri et al. 1998), these populations are more severely threatened than previously considered (Oregon Natural Heritage Program 2001). Therefore, attention should be paid to maintaining high population sizes and protecting pollinator faunas. High inbreeding depression in these population and in others (e.g., the rare *Silene douglasii* var. *oraria*, Kephart et al. 1999; and the rare *Astragalus linifolius*, Karron 1989) emphasize that rare species, contrary to some arguments (Lande and Schemske 1985, and Schemske and Lande 1985), can be subject to severe levels of this natural genetic threat.

Summary –

I describe the magnitude and timing of inbreeding depression in 14 populations (seven species) in the *M. moschatus* alliance and addressed three main questions. 1) I found that inbreeding depression was weakly associated with anther-stigma separation, outcrossing rates, pollen/ovule ratio, corolla size, and autonomous seed set, providing some support to the hypothesis of a positive association between fitness and "selfing" alleles among populations and species (Holsinger 1988, Uyenonyama and Waller 1991a, b, Takebayashi and Delph 2000). However, the relationship was not strong, and selective forces other than inbreeding depression are likely important in the evolution of plant mating systems. 2) Taxa inherited a significant portion of both mating-system traits and to a much lesser degree, inbreeding depression from their ancestors. In this group, the evolution of mating system and inbreeding depression is evident above the population-level. Mating system (both outcrossing rates and floral morphology) appears to be more evolutionary constrained than is inbreeding depression. I suggest that the occurrence of random mutations of deleterious alleles of large effect may be largely responsible for the current distribution of inbreeding-depression levels in this group. 3) There was no clear sequence of evolution of mating system and inbreeding depression, and no evidence that inbreeding depression promotes the evolution of outcrossing, nor that selfing reduces inbreeding depression, at least over the time scale reflected in this phylogeny. Last, outcrossing rates were found to be substantially more evolvable than floral traits, supporting the notion that ecological factors can be as, or more important than morphological features in determining mating system evolution.

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CHAPTER 4. THE RELATIONSHIP BETWEEN INBREEDING DEPRESSION AND OUTBREEDING DEPRESSION IN 14 POPULATIONS OF THE *MIMULUS MOSCHATUS* ALLIANCE

ABSTRACT

Inbreeding and outbreeding depression are important forces influencing population fitness parameters and molding evolution of mating systems. Despite much investigation, the relationship between these two factors remains ambiguous. I compared the relative performance of selfed plants to between population- and within population-outcrossed plants (inbreeding depression) and the relative performance of within population- to between population-outcrossed plants (outbreeding depression) in 14 populations of seven closely related species in the *Mimulus moschatus* alliance. Species and populations that suffer from high inbreeding depression were more likely to suffer from high outbreeding depression, while species with little or no inbreeding depression displayed little outbreeding depression or even fitness increases when crossed with individuals from another population. While inbreeding depression was positively correlated with outcrossing rates, outbreeding depression had no relationship with outcrossing rates. Phylogenetic inertia was weak for inbreeding and outbreeding depression, but not for mating-system traits. This indicated that levels of inbreeding and outbreeding depression fluctuate on a finer evolutionary scale than does mating system. Interpretations of the genetic basis of inbreeding and outbreeding depression and their relation to mating-system evolution are discussed. Last, mediation of negative fitness consequences of extreme crossing distances is discussed in relation to conservation and restoration biology.

INTRODUCTION

The degree of genetic relatedness between mates can have severe fitness consequences to their offspring. At one end of the spectrum, very closely related mates can result in low fitness (inbreeding depression) and at the other end, very distantly related mates can also result in low fitness (outbreeding depression). It generally accepted that some intermediate level of genetic similarity of mates results in the highest fitness (Waser 1993, Lynch and Walsh 1998, Montavlo and Ellstrand 2001).

The association of genetic relatedness to offspring fitness has received much attention in relation to its role in the evolution of mating systems (Fisher 1941, Lande and Schemske 1985, Schemske and Lande 1985, Charlesworth and Charlesworth 1987, Holsinger 1988, Husband and Schemske 1996) and in biological conservation (Templeton 1986, Karron 1989, Barrett and Kohn 1991, Van Treurnen, et al. 1993, Fenster and Dudash 1994, Newman and Pilson 1997, Saccheri et al. 1998). Additionally, debates have centered around the relative importance of two fitness components affected by long-distance crosses: adaptation to the local ecological versus genetic environments (Lynch and Walsh 1998, Edmands 1999, Fenster and Galloway 2000, Montavlo and Ellstrand 2001). Despite individual attention to fitness consequences of mating at both spectra of relatedness, the relationship between near and distant crosses remains ambiguous (Schierup and Christiansen 1996, Lynch and Walsh 1998). The relationship between the two can have important implications for understanding the genetic basis of inbreeding and outbreeding depression and for conservation and restoration biology.

Background on inbreeding depression –

The reduction in vigor of offspring from closely related mates relative to offspring from unrelated mates (inbreeding depression) is a widespread phenomenon that has been observed in both cultivated (e.g., Neal 1935, Wright 1977) and in natural plant populations (e.g., Schemske 1983, Ritland and Ganders 1987, Karron 1989, Dudash

1990, Ågren and Schemske 1993, Kephart et al. 1999, Takebayashi and Delph 2000). Inbreeding depression can be expressed at different life-history stages, from embryo formation and seed germination, to survival and life-time fecundity. Generally, more outcrossing taxa express inbreeding depression both in early and late life-stages, while selfing taxa expressing inbreeding depression primarily in later life-stages (Husband and Schemske 1996).

The degree of inbreeding depression expressed appears to be related to the environment under which the offspring are raised (Dudash 1990, Lynch and Walsh 1998). The conventional wisdom is that higher environmental stress increases the selection coefficient, resulting in increased expression of inbreeding depression (Walsh unpublished manuscript). However, the evidence for this is inconsistent. Dudash (1990) and Eckert and Barrett (1994) observed greater inbreeding depression in field relative to more benign greenhouse environments, but Holtsford and Ellstrand (1989) observed the opposite result. It is likely that the expression of inbreeding depression to environmental stress is a convex function, with low inbreeding depression in benign and extremely severe environments (Walsh unpublished manuscript). Increasing stress magnifies inbreeding depression, but beyond some point, stress will kill so many individuals that the variance, and thus the opportunity for selection, will decrease.

Genetic basis of inbreeding depression –

There are two primary, non-mutually exclusive, models of the genetic basis of inbreeding depression. In the partial dominance model, fitness decline in inbred offspring is due to recessive or partly recessive deleterious alleles expressed at homozygous loci. In the overdominance model, fitness decline is due to heterozygote advantage, where the heterozygote has greater fitness than either homozygote (see review in Charlesworth and Charlesworth 1987). The determination of the genetic mechanism is difficult because the two mechanisms may occur at the level of single loci or many loci (Moll et al. 1964). Across taxa, experimental evidence appears to be most compatible with the partial dominance model of inbreeding depression (Moll et al. 1964, Simmons and Crow 1977,

Mather and Jinks 1982, Crow and Simmons 1983, Jinks 1983, Sprague 1983, Charlesworth and Charlesworth 1987, Crow 1993, Mitton 1993, Fenster and Dudash 1994, Dudash and Carr 1998). However, Mitton (1993) presented evidence for overdominance in natural populations of three species: ryegrass (*Lolium multiflorum*), common killfish (*Fundulus heteroclitus*), and in a laboratory population of Atlantic cod (*Gadus morhua*). Also, most of the additional studies supporting the partial dominance theory involved crop species that have been subjected to strong artificial selection on additive genes, potentially biasing the importance of partial dominance (Comstock and Robinson 1948, Robinson et al. 1949, Moll et al. 1964, Fenster and Dudash 1994). Studies on *Drosophila* indicate that half of the inbreeding depression is caused by nearly completely recessive lethal alleles and the remainder is caused by partially recessive mildly deleterious alleles (see review in Lewontin 1974, Simmons and Crow 1977, Crow and Simmons 1983, Charlesworth and Charlesworth 1987). Studies on *Mimulus guttatus* also support the partial dominance model, however with many genes of small effect rather than a few genes of large effect (Dudash and Carr 1998a, 1998b, Willis 1999). Further, the presence of inbreeding depression in primarily selfing taxa suggests that many loci of small effect is the most plausible explanation in general, since they are much more difficult to purge (Charlesworth and Charlesworth 1987, Ågren and Schemske 1993).

The genetic basis of inbreeding depression has important implications to the evolution of mating system and conservation biology. If inbreeding depression is caused by overdominance then the genetic load cannot be purged through a period of selfing. Likewise, if inbreeding depression is caused by many deleterious loci of small effect, population fitness will not improve after a period of enforced selfing (Lande and Schemske 1985, Holsinger 1988, Schultz and Willis 1995). However, if inbreeding depression is caused by partial dominance with a few loci of large effect, than a period of enforced selfing can result in a purging of deleterious alleles. Thus, various conservation and evolutionary consequences follow from the alternative theories of the genetic basis of inbreeding depression. For example, through a controlled breeding program, inbreeding

depression was reduced in a captive population of the rare, Speke's gazelle (*Gazella spekei*; Templeton and Read 1984). However, based on evidence that inbreeding depression was caused by many alleles of small effect, Willis (1999) warned that attempts to reduce inbreeding depression in some populations would be unsuccessful. A reduced genetic load has been shown to reduce population and meta-population extinction probabilities (Newman and Pilson 1997, Saccheri et al. 1998).

Background on outbreeding depression –

Outbreeding depression is the reduction in fitness of offspring from genetically or geographically distant crosses relative to close, generally within-population, crosses. The fitness reduction can be observed in the F1, F2, backcross, or later generations. Generally, outbreeding depression is observed on the scale of between population crossing. However, there is some evidence of within population-level outbreeding depression (Price and Waser 1979). A number of studies of native plants have observed this phenomenon (see review in Levin 1978, Waser 1993).

Genetic basis of outbreeding depression –

There are two proposed modes of gene action to explain the genetic basis of outbreeding depression (Price and Waser 1979, Lynch 1991, Waser 1993, Schierup and Christiansen 1996, Montalvo and Ellstrand 2001). The "ecological mechanism" proposes that plants, which are adapted to their local environment, suffer when their genes are diluted with foreign genes, adapted to an alternative environment. In the F1 generation, between-population crossing leads to a 50 % dilution of the locally adapted genome. This mechanism is revealed if parental populations are shown to be locally adapted (i.e., there is a genotype X environment interaction) and F1 hybrid progeny perform worse than the parental mean when tested together in the parental environments (Montavlo and Ellstrand 2001). The "genetic mechanism" proposes that populations become adapted to the genetic as opposed to the ecological environment. Restricted gene flow between and drift within populations creates coadapted gene complexes via epistatic interactions (Moll et

al. 1965, Campbell and Waser 1987, Mitton 1993). Between-population crossing disrupts the positive epistatic interactions and fitness subsequently declines. Under this model, the greatest fitness decline should occur after segregation in the F2 generation. A second mechanism for genetic outbreeding depression is underdominance at a number of loci (Ritland and Ganders 1987, Lynch 1991, Schierup and Christiansen 1996). This mechanism has full effect in the F1 generation, resulting from intralocus interaction rather than epistasis.

Alternatively, between-population crosses can result in an increase in vigor over parental values, generally referred to as heterosis, or hybrid vigor. Crossbreeding of two separate lines has long been employed by plant and animal breeders for its beneficial effects (Darwin 1876, Sprague 1983). However, often when heterosis is observed in the F1 generation, much of it is lost in subsequent generations (Lynch and Walsh 1998). Occasionally, the fitness of F2 and later generations, drops below the fitness level of the original parental lines. Crosses between different species or remotely related populations often results in reduction or complete loss of fitness (i.e., outbreeding depression).

The response of progeny fitness to increasing genetic distance between parents suggests that there is a shift in predominant gene interaction as mates become more distantly related (Lynch and Walsh 1998). The primary cause of inbreeding depression is generally believed to be partial dominance, while the decline in fitness under outcrossing is usually attributed to the breakup of coadapted gene complexes in the parental populations. Thus, Lynch and Walsh (1998) stress there is a shift in emphasis from interactions within loci (dominance) to those among loci (epistasis).

Relationship between inbreeding depression and outbreeding depression –
While inbreeding depression and outbreeding depression (or its converse, heterosis) might appear to be unrelated, since their mechanisms may be different, they may be non-causally correlated through mating system. Thus a spurious relationship may be expected between the two, because both inbreeding depression and outbreeding depression are believed to evolve in response to alterations in mating system. Fenster and Dudash

(1994) proposed that outbreeding depression should be greatest for species with high selfing rates. The reason for this is that the amount of recombination determines the extent to which linkage disequilibrium can be formed between loci with fitness interactions (Lewontin 1974, Fenster and Dudash 1994). When recombination is limited, or the approach to linkage equilibrium is decreased by selfing, epistatic gene complexes can be formed rapidly. Thus, outbreeding depression is expected to be greatest for species with high selfing rates (Fenster and Dudash 1994). Additionally, if outbreeding depression is due to interactions within loci (underdominance) the fitness decrease is expected to be greatest in small and selfing populations (Schierup and Christiansen 1996). Populations or species with high selfing rates are also expected to have low inbreeding depression (see Lande and Schemske 1985, Schemske and Lande 1985, Charlesworth and Charlesworth 1987, and Husband and Schemske 1996) therefore, a negative relationship between inbreeding and outbreeding depression is expected.

Alternatively, some theory predicts that highly selfing populations should have low outbreeding depression or express heterosis. Lynch et al. (1995) predicted that genetic degradation proceeds more rapidly in selfing populations of finite size, due to fixation of deleterious alleles. Therefore, highly selfing populations cannot purge their genome, since the deleterious alleles do not segregate. Thus, crossing between populations fixed for various different deleterious recessive alleles would result in a fitness advantage over crossing within populations (i.e., heterosis).

In the present study, I address the relationship between inbreeding depression and outbreeding depression in 14 populations of the *Mimulus moschatus* alliance, using a phylogenetic comparative method. Specifically, I test the hypothesis that inbreeding depression in this group is caused by partially recessive deleterious alleles (i.e., the partial dominance model) and outbreeding depression is caused by adaptation to the genetic environment (i.e., epistatic interactions or underdominance). Inbreeding depression and outbreeding depression are predicted to correlate with outcrossing rates in opposite directions. Inbreeding depression should be greatest for populations with high outcrossing rates due to a higher genetic load of deleterious recessive alleles (Lande and

Schemske 1985, Charlesworth and Charlesworth 1987). While, outbreeding depression should be greatest for populations with low outcrossing rates since the coadapted gene complexes and underdominant loci become fixed are more likely to form in highly selfing relative to more outcrossing populations (Lewontin 1974, Fenster and Dudash 1994). Therefore, as suggested by Fenster and Dudash (1994), a negative correlation should exists between inbreeding depression and outbreeding depression due to the linking factor of mating system coupled with the various modes of gene action. Additionally in these seven population pairs, I test for the presence of heterosis, or fitness advantage of mean between population crosses to mean within population crosses.

To address these hypotheses I use the *M. moschatus* alliance (Scrophulariaceae) as a study system. By integrating data on inbreeding depression, outbreeding depression, and outcrossing rates, with phylogenetic information, I explore the historical relationship among these traits and the effect of ancestry. Understanding the link between inbreeding and outbreeding depression is essential to help identify the underlying genetic mechanisms, to predict fitness consequences of crossing individuals within and among populations, and to have an understanding of what spatial scale crosses between populations result in outbreeding depression.

METHODS

Study organisms—The *Mimulus moschatus* alliance is a clade of 12 moderate to small-flowered, viscid species, with a broad range of outcrossing rates ($t = 0.00 - 0.69$; see Chapter 2). All members of the alliance are self-compatible, and most will autonomously self-pollinate in the absence of insect visitation. Three species in the alliance are very widespread, one is moderately widespread, and eight of the twelve species are extremely geographically restricted, prompting attention from conservation organizations (Oregon Natural Heritage Program 2001). Additionally, most of the species occur in small (i.e., fewer than 1,000 individuals, Carlson unpubl. data) isolated populations.

All populations were located over two kilometers apart, and most were over ten (Figure 4-1). Distances of these magnitudes have been large enough to generate fitness differences in between-populations crosses of *Chamaecrista fasciculata* (Fenster and Galloway 2000). Additionally, most plant populations have quite restricted neighborhood sizes and high population subdivision (Levin 1981, Fenster 1991, Karron et al. 1995), so that crosses between plants separated by just tens of meters should result in mixing of reasonably divergent gene pools. The species included in the analysis fall into three clades: *M. ampliatus* + *M. hymenophyllus* + *M. patulus*; *M. washingtonensis* + *M. jungermannioides*; and *M. dudleyi* + *M. floribundus* (Whittall, et al. in press). Figure 4-2 shows the strict-consensus composite tree (i.e., supertree, sensu Bininda-Emonds and Sanderson 2001). The supertree is based on two chloroplast, two nuclear, and a mating system-independent morphological data sets (see Chapter 1). Supertree analysis was used rather than total evidence because taxon sampling was not consistent among the data sets. Supertree analysis does not include information about branch lengths. Therefore, all branches were assigned equal lengths, except population-level branches that were given lengths half those of others. This decision was made since branch lengths from ITS and chloroplast species trees tended to be quite similar (Whittall et al. unpublished manuscript, Beardsley unpublished manuscript), and morphologically the populations within species showed some differentiation, but not worthy of species-level branch lengths (see Chapter 2).

Inbreeding and outbreeding depression estimation –

I grew individuals from field-collected seed, of 60-80 randomly selected, mature plants per population. Seeds from *M. ampliatus*, *M. hymenophyllus*, *M. patulus*, and *M. washingtonensis* were cold-stratified in the dark at 4^0C for approximately 70 d, prior to

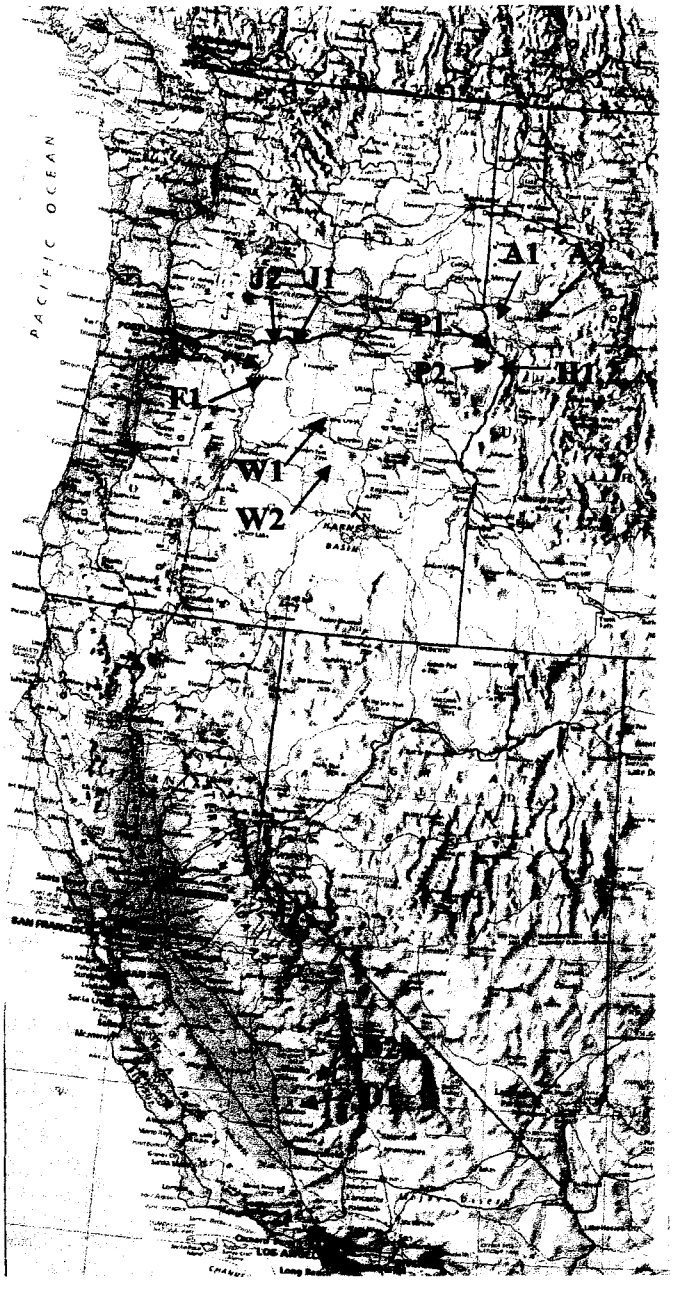

FIGURE 4-1. Distribution of source populations for estimating inbreeding and outbreeding depression levels. A1 and A2 = *M. ampliatus* populations 1 and 2, D1 and D2 = *M. dudleyi* populations 1 and 2, F1 and F2 = *M. floribundus* populations 1 and 2, H1, H2 = *M. hymenophyllus* populations 1 and 2 (2 km apart from one another), J1 and J2 = *M. jungermannioides* populations 1 and 2, P1 and P2 = *M. patulus* populations 1 and 2, W1 and W1 = *M. washingtonensis* populations 1 and 2.

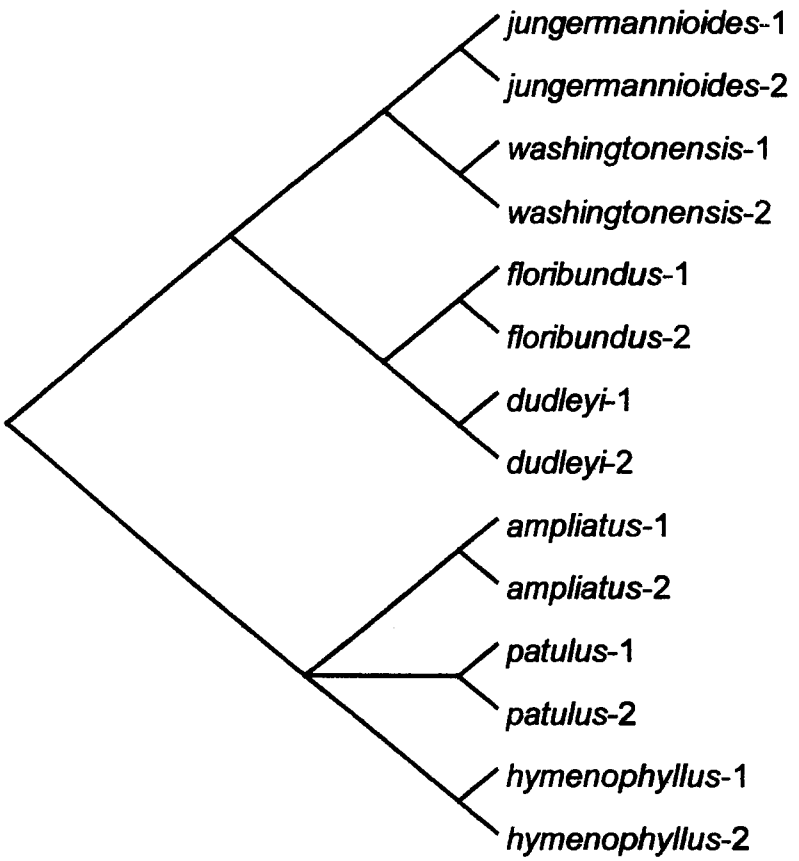

FIGURE 4-2. The strict-consensus supertree for the *Mimulus moschatus* alliance. Four species in the *Mimulus moschatus* alliance without inbreeding and outbreeding depression estimates are pruned from the tree (see Chapter 1 for a complete tree). Branch lengths were assumed equal, except terminal branches to populations, which are half the length of all others.

germination. All seeds were germinated on moist paper in plastic zip-lock bags under 16 h day length and 22°C in the University of Trondheim, Norway, Botany Department greenhouse. Upon germination, a single randomly selected seedling per family was transferred to its own 730 cm^3 pot, with standard potting mix ("Elg P-Jord:" 50 % humus, 25 % sand, 25 % perlite). All plants were watered from below as needed; however no fertilizer was added to the soil during the study. All species were grown in the greenhouse from November 1998 to February 1999, under the same conditions.

Only one individual per family was used to generate selfed and outcrossed offspring. I performed two types of cross-pollinations: crosses between individuals of the same population (OW crosses) and crosses between individuals of another population (OB crosses). All crosses were reciprocal, such that each individual served as both a pollen donor and pollen recipient to each crossing partner (see Lynch 1988). Because few families were represented, multiple crosses per individual were made in an incomplete diallel, for both populations of *M ampliatus*. OW and OB crosses were paired with self pollinations (SELFW and SELFB). Normally, these species produce two flowers per node and self and outcross pollinations were paired on these nodes to reduce positional effects (Latta and Ritland 1994, Carr and Dudash 1996). In cases where only one flower per node was produced or if the two flowers were too developmentally disparate, flowers were chosen haphazardly. OW and OB crosses were performed on different nodes. All species were emasculated prior to anthesis. The two small-flowered species, *M. patulus* and *M. floribundus*, were emasculated while in bud, about one to two days prior to opening, because anther dehiscence occurs just prior to the flower opening. All experimental flowers were emasculated either one day prior to, or the day of, floral opening. Ten to 15 control flowers per population were emasculated to confirm that no pollen contamination occurs. No mature seeds were observed in any of the control emasculations. Flowers were pollinated two hours to one day following emasculation to allow the stigma to become receptive and the lobes to reopen after being touched. *Mimulus* stigmas are thigmatropically sensitive and will reopen if insufficient pollen is deposited (Newcombe 1922, Meinke 1992, Fetscher and Kohn 1999). Hand pollinations

were performed by brushing a single mature anther against the stigma until the lobes closed around the anther. For the four populations in the *M. dudleyi* + *M. floribundus* clade, 43 to 48 plants per population were crossed, and in the four populations in the *M. jungermannioides* + *M. washingtonensis* clade, 57 to 62 plants per population were crossed. The six populations in the *M. ampliatus* + *M. hymenophyllus* + *M. patulus* clade had poor germination and as a result between six and 18 maternal plants per population were crossed.

Fruits were collected three to five weeks following pollination, as they became dry, but before dehiscence. Ten or 20 seeds per capsule (or less if fewer seeds were set) were germinated in similar conditions to their parents. Selfed and outcrossed offspring from each set of reciprocal crosses were grown together in the Oregon State University, Botany Department, greenhouse in one of three blocks under conditions similar to their parents from June 1999 to September 2000. Five germinated seeds were randomly selected and planted in pots (ca. 730 cm^3) with a standard potting mix ("Witham Farms Standard Mix:" 50 % humus, 25 % sand, 25 % perlite). A total of 3240 individuals were planted. After 60 d, the percentage of germinated seeds was scored.

Offspring were grown with 16 h light at 22°C and 8 h dark at 15°C. They were watered from below as needed, but not fertilized. Six of the seven species are annuals, and they were allowed to grow until they naturally senesced. *Mimulus jungermannioides* is a perennial, and it was not watered after the last of the other annuals senesced, i.e., after ca. 105 d. In addition to number of seeds per maternal fruit and germination percent, I measured offspring survival and total number of flowers per plant. These measures together seem to be a good estimate of fitness over a single generation (Latta and Ritland 1994, Kephart et al 1999). They include components of multiple life-history stages: embryo and seedling fitness (seed set and germination percent) and mid-life and lifetime fecundity (survival and flower number). Cumulative fitness was defined as seed number times germination percent times survival times flower number.

Analysis of Data –

To estimate levels of inbreeding depression, cumulative fitness differences between pollination treatments were compared within populations, using the "relative performance" (RP_{WS}) for each experimental unit (a self-outcross within population pair) following the method proposed by Ågren and Schemske (1993). The measurement $RP_{WS} = (W_{OW} - W_S)/\text{maximum}\ (W_{OW}, W_S)$, where W_{OW} is the cumulative fitness for a within population cross and W_S is the cumulative fitness of a selfed cross. Positive RP_{WS} values indicate that the performance of outcrossed individuals exceeds that of selfed individuals (inbreeding depression). Negative values indicate that selfed individuals outperform outcrossed individuals (within population outbreeding depression). +1 and –1 bound this measure. For positive values, RP_{WS} is identical to the traditional measure of inbreeding depression: $\delta = 1 - (\text{self/outcross})$. However, the traditional measure is bounded by +1 and $-\infty$ and if negative values occur is weighted towards within population outbreeding depression when summed (Carr et al. 1997).

Additionally, I measured the cumulative fitness difference between outcrossed between population relative to selfed offspring. This was measured as $RP_{BS} = (W_{OB} - W_S)/\text{maximum}\ (W_{OB}, W_S)$, where W_{OB} is the cumulative fitness for a between population cross and W_S is the cumulative fitness of a selfed cross. Positive RP_{BS} values indicate that the performance of between population outcrossed individuals exceeds that of selfed individuals.

Outbreeding depression was measured as the cumulative fitness of between population crosses relative to within population outcrosses. Outbreeding depression was estimated using a relative performance measure of $RP_{WB} = (W_{OW} - W_{OB})/\text{maximum}\ (W_{OW}, W_{OB})$, where W_{OW} is the cumulative fitness for a between population cross and W_{OB} is the cumulative fitness of a within population cross. Positive RP_{WB} values indicate that the performance of within population-crossed individuals exceeds that of between population-crossed individuals (between population outbreeding depression). Negative values indicate that between population-crossed individuals outperform within

population-crossed individuals (population-level hybrid vigor). +1 and –1 bound this measure.

Heterosis is indicated when mean between population fitness is greater than the mean within population fitness. Therefore to test for the presence of heterosis, the mean RP_{WB} for each pair of parents used in the reciprocal crosses between populations was calculated. This value is calculated for species.

I analyzed each population separately using SPSS Base 8.0. Differences in relative performance were analyzed with one sample *t*-test was used to determine whether RP_{WS}, RP_{BS}, and RP_{WB} differed significantly from zero. Population-wise Bonferroni correction was used to maintain the probability of committing type I error at 0.05. Species level-heterosis was analyzed by one sample *t*-tests against zero. Differences among species were analyzed with nested ANOVA (populations within species).

To test whether there is a negative relationship between inbreeding depression and outbreeding depression, and to test if these factors are related to mating system, correlation analysis was used in two ways: on phylogenetically "naïve" and phylogenetically corrected data. The results of the phylogenetically naïve and corrected data were compared to interpret the extent of phylogenetic effect and the nature of the phylogenetic signal (see Armbruster et al. 2002). Felsenstein's (1985) independent contrasts were used using the computer program CAIC (Purvis and Rambaut 1995) to achieve a phylogenetically corrected analysis. Additionally, Phylogenetic Mixed Model analysis was used to estimate the relative importance of phylogeny in explaining the phenotypic variation (Lynch 1991b) in Martin's (2001) computer program, COMPARE version 4.4. In this model, "phylogenetic heritability" (H^2_P) can be estimated, with values near one indicating that resemblance among relatives is high, and values near zero indicating that phylogenetic intertia is minimal (Lynch 1991, Martins 2001).

RESULTS

Both inbreeding depression and outbreeding depression values (RP_{WS} and RP_{WB}, respectively) varied widely among species (Table 4-1), but means were significantly heterogeneous only for inbreeding depression (Table 4-2, see also Chapter 3). Inbreeding depression ranged from around –0.13 to 0.53 with an average of 0.20. Thus, selfing reduced fitness by about 20 % on average for these populations. Outbreeding depression ranged from –0.36 to 0.23 with an average of –0.07. This indicates a trend for fitness to be enhanced by 7 % on average by crossing between populations relative to within populations. The fitness of between population relative to self crosses (RP_{BS}) were nearly always moderately to strongly positive, indicating that between population crosses were beneficial over selfing at least in the F1 generation. RP_{BS} values ranged from –0.05 to 0.53. Species level-heterosis was slightly positive in almost all cases, but was significantly greater than zero only for one species (*M. jungermannioides*), which inter-population hybrids had an average 18 % fitness advantage over within population outcrossed plants (Table 4-3).

Populations with high within-population outcross fitness relative to self fitness did not appear to have corresponding high between-population outcross fitness relative to self fitness. Thus, the two measures of self to outcross fitness, RP_{WS} and RP_{BS}, did not appear to be related (Table 4-4, Fig. 4-3).

The expected equilibrium levels of inbreeding depression as a function of outcrossing rate, are mapped over the experimental results in Figure 4-4. This model assumes inbreeding depression is caused by mutations at unlinked loci without epistasis (Charlesworth et al. 1990).

Inbreeding depression and outbreeding depression values tended to be positively correlated (Table 4-4, Fig. 4-5), although the correlation was only marginally statistically

TABLE 4-1. Mean inbreeding depression, outbreeding depression, outcrossing rate (*t*), and anther-stigma separation for 14 populations in the *M. moschatus* alliance. Inbreeding and outbreeding depression are measured as relative performance (Ågren and Schemske 1993). RP_{WS} = relative performance of OW to SELF (i.e., inbreeding depression), RP_{BS} = relative performance of OB to self; positive values indicate that outcrossed progeny outperform selfed progeny. RP_{WB} = relative performance of OW to OB (outbreeding depression); positive values indicate that within-population outcrossed individuals outperform between-population outcrossed individuals. Means are presented above standard errors. Inbreeding and outbreeding depression values significantly different from 0.0 are indicated as † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$.

Species (population)	RP_{WS}	RP_{BS}	RP_{WB}	*t*	anther-stigma separation (mm)
M. ampliatus-1	0.241	0.247	-0.054	0.00	0.61
	(0.204)	(0.165)	(0.193)		(0.09)
M. ampliatus-2	0.181	0.161	+0.108	0.06	0.70
	(0.113)	(0.209)	(0.212)		(0.09)
M. dudleyi-1	0.468**	0.506**	-0.152	0.41	1.08
	(0.116)	(0.094)	(0.116)		(0.09)
M. dudleyi-2	0.459**	0.167	+0.228†	0.47	1.12
	(0.115)	(0.104)	(0.097)		(0.08)
M. floribundus-1	-0.109	0.164	-0.039	0.00	-0.16
	(0.172)	(0.138)	(0.152)		(0.04)
M. floribundus-2	0.145	-0.048	-0.029	0.06	-0.13
	(0.124)	(0.134)	(0.121)		(0.06)
M. hymenophyllus-1	-0.129	0.526**	-0.300†	0.44	0.13
	(0.229)	(0.120)	(0.160)		(0.05)
M. hymenophyllus-2	0.347	0.100	-0.033	0.40	-0.02
	(0.237)	(0.188)	(0.186)		(0.08)

TABLE 4-1 continued. Mean inbreeding depression, outbreeding depression, outcrossing rate (t), and anther-stigma separation for 14 populations in the *M. moschatus* alliance. Inbreeding and outbreeding depression are measured as relative performance (Ågren and Schemske 1993). RP_{WS} = relative performance of OW to SELF (i.e., inbreeding depression), RP_{BS} = relative performance of OB to self; positive values indicate that outcrossed progeny outperform selfed progeny. RP_{WB} = relative performance of OW to OB (outbreeding depression); positive values indicate that within-population outcrossed individuals outperform between-population outcrossed individuals. Means are presented above standard errors. Inbreeding and outbreeding depression values significantly different from 0.0 are indicated as † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$.

Species (population)	RP_{WS}	RP_{BS}	RP_{WB}	t	anther-stigma separation (mm)
M. jungermannioides-1	0.042	0.240**	-0.159†	0.30	0.41
	(0.096)	(0.084)	(0.086)		(0.06)
M. jungermannioides-2	0.213†	0.491**	-0.225*	0.45	0.41
	(0.106)	(0.077)	(0.101)		(0.09)
M. patulus-1	0.218	0.279	-0.003	-	-0.25
	(0.239)	(0.200)	(0.241)		(0.03)
M. patulus-2	-0.027	0.014	-0.059	0.09	-0.17
	(0.215)	(0.161)	(0.200)		(0.03)
M. washingtonensis-1	0.172	0.441**	-0.362**	0.60	0.88
	(0.121)	(0.086)	(0.092)		(0.12)
M. washingtonensis-2	0.529**	0.307**	+0.138	0.69	0.77
	(0.073)	(0.085)	(0.098)		(0.13)

TABLE 4-2. Nested ANOVA of A) inbreeding (RP_{WS}) and B) outbreeding depression (RP_{WB}) in the *M. moschatus* alliance. Populations are nested within species.

A.) Inbreeding Depression

Source	df	MS	F	P
Species	7	2.996	5.211	0.022
Populations within species	7	0.575	1.560	0.147
Error	276	0.368		

B.) Outbreeding Depression

Source	df	MS	F	P
Species	7	0.857	0.626	0.724
Populations within species	7	1.333	2.829	0.007
Error	440	0.484		

TABLE 4-3. Species level-heterosis for seven species in the *M. moschatus* alliance. Positive values indicate that the mean fitness of between-population outcrossed individuals outperform within-population outcrossed individuals (i.e., heterosis). Means and (SE) are presented in the second column. n = the number of family pairs. Values significantly different from 0.0 are indicated as † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$.

Species	Heterosis	n
M. ampliatus	0.049 (0.095)	34
M. dudleyi	-0.044 (0.085)	48
M. floribundus	0.016 (0.093)	40
M. hymenophyllus	0.219 (0.126)	15
M. jungermannioides	0.184 (0.068)**	60
M. patulus	0.059 (0.158)	22
M. washingtonensis	0.093 (0.071)	57

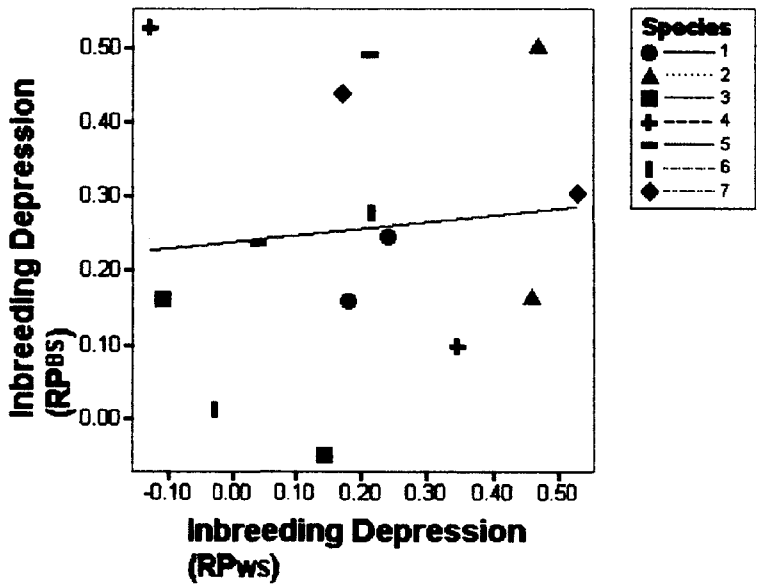

FIGURE 4-3. Fitness relationship of within-population outcrossed to selfed and between-population outcross to selfed offspringl. RP_{WS} = within-population outcrossed to selfed and RP_{BS} = between-population outcross to selfed offspring. $R = 0.102$, $P = 0.730$, $n = 14$. Species 1 = *M. ampliatus*, 2 = *M. dudleyi*, 3 = *M. floribundus*, 4 = *M. hymenophyllus*, 5 = *M. jungermannioides*, 6 = *M. patulus*, 7 = *M. washingtonensis*.

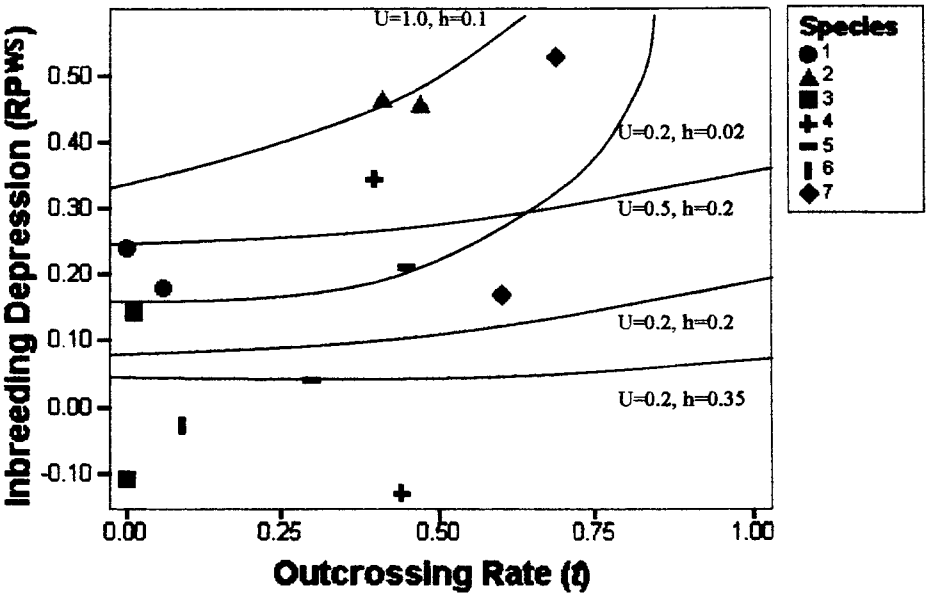

FIGURE 4-4. Mean population inbreeding depression (RP_{WS}) and outcrossing rates, showing data and theory. Data points are from the 14 *M. moschatus* alliance populations in the study. Lines indicate the predicted effects of mutation rate per sporophyte genome, *U*, and dominance, *h*, on the equilibrium levels of inbreeding depression, assuming unlinked loci. Lines were created by interpolating between the simulation results in Charlesworth et al. (1990). Species 1 = *M. ampliatus*, 2 = *M. dudleyi*, 3 = *M. floribundus*, 4 = *M. hymenophyllus*, 5 = *M. jungermannioides*, 6 = *M. patulus*, 7 = *M. washingtonensis*.

significant in the phylogenetically naïve analysis ($R = 0.47$, $P < 0.10$). Populations with high levels of inbreeding depression were more likely to possess moderate levels of outbreeding depression. Populations with little or no inbreeding depression often expressed either no outbreeding depression, or moderate heterosis.

While inbreeding depression (both RP_{WS} and RP_{BS}) was weakly, positively related to outcrossing rates, there was no relationship between outbreeding depression (RP_{WB}) and mating system (Table 4-4, Fig. 4-6).

Phylogenetic effect –

Phylogenetic history was relatively unimportant in explaining the magnitude of both inbreeding and outbreeding depression relative to mating system and floral traits. While phylogenetic heritabilities (Lynch 1991) of mating system traits all approached 1.00 (see Chapter 3), inbreeding depression and outbreeding depression had much lower phylogenetic heritabilites. $H^2_P = 0.363$ and 0.054 for inbreeding depression and outbreeding depression, respectively. Values that approach zero indicate evolutionary lability, in which trait inheritance from ancestry is minimal and values for taxa can be assumed independent (Lynch 1991, Martins 2001). Because of high evolutionary lability, correlation coefficients of phylogenetically corrected inbreeding and outbreeding values were all of similar magnitudes and directions to phylogenetically naïve correlations, and in some cases the relationships were even stronger after phylogenetic correction (Table 4-4). Stronger phylogenetically corrected correlations indicates "phylogenetic lag" (i.e., the phylogenetic signal retarding response to selection; see Armbruster et al. 2002). Fine scale (within species) contrasts of inbreeding depression and outbreeding depression were of a similar magnitude as higher-taxonomic level contrasts (RP_{WS}: $t = 0.043$, $df = 9$, $P > 0.50$; RP_{WB}: $t = 1.132$, $df = 9$, $P > 0.30$, Fig. 4-6). However, for mating system-traits, contrasts in outcrossing rates were significantly greater among higher levels relative to

TABLE 4-4. Correlations of inbreeding depression, outbreeding depression, and mating system indicators among 14 populations (seven species) in the *M. moschatus* alliance. RP_{WS} = relative performance of OW to SELF (i.e., inbreeding depression), RP_{BS} = relative performance of OB to self. RP_{WB} = relative performance of OW to OB (outbreeding depression). Phylogenetically corrected correlations are represented below the diagonal; $n = 11$ contrasts. † = $P < 0.10$, * = $P < 0.05$, ** = $P < 0.01$.

	RP_{WS}	RP_{BS}	RP_{WB}	Outcrossing rate (*t*)	Anther-stigma separation
RP_{WS}	-	0.102	0.473†	0.502†	0.631*
RP_{BS}	-0.349	-	-0.592*	0.614*	0.445
RP_{WB}	0.441	-0.769**	-	-0.183	0.100
t	0.452	0.410	-0.161	-	0.524†
Anther-stigma separation	0.365	0.331	-0.085	0.781**	-

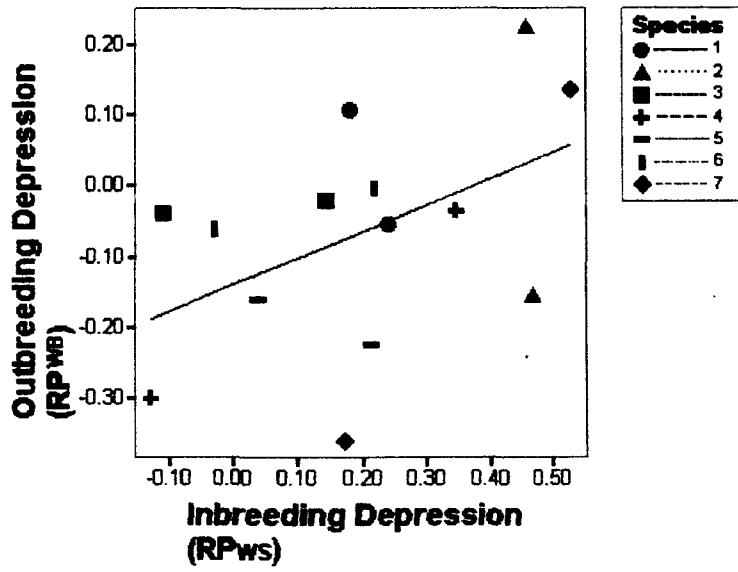

FIGURE 4-5. Relationship between mean inbreeding and outbreeding depression in the *M. moschatus* alliance. Inbreeding depression is measured as RP_{WS}, i.e., relative performance of (outcross within population – self)/max. Outbreeding depression is measured as RP_{WB}, i.e., relative performance of (outcross within population – outcross between population)/max. Species 1 = *M. ampliatus*, 2 = *M. dudleyi*, 3 = *M. floribundus*, 4 = *M. hymenophyllus*, 5 = *M. jungermannioides*, 6 = *M. patulus*, 7 = *M. washingtonensis*.

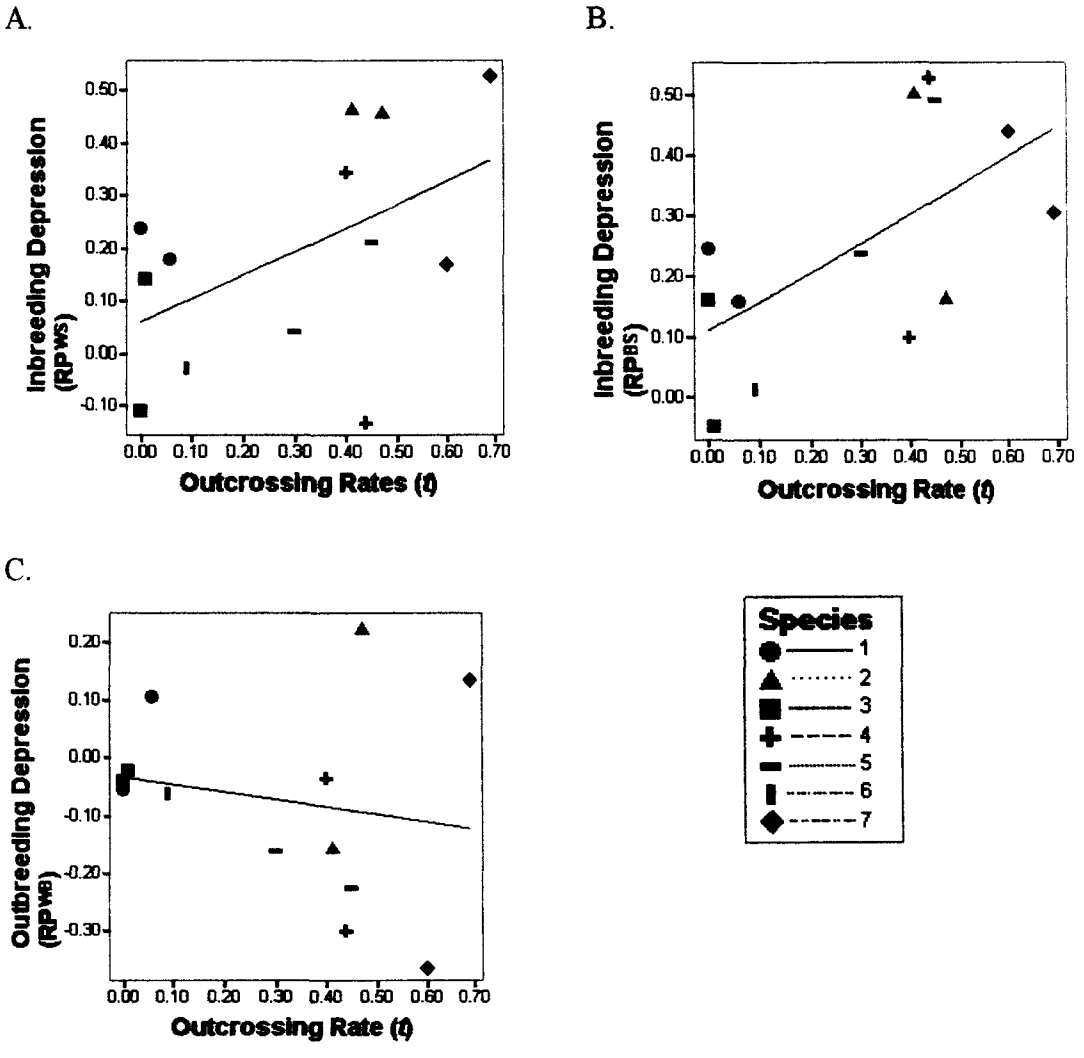

FIGURE 4-6. Relationship between mean inbreeding depression and outcrossing rate, and between outbreeding depression and outcrossing rate. A) shows inbreeding depression measured as RP_{WS}, i.e., relative performance of (outcross within population – self)/max. B) shows inbreeding depression measured as RP_{BS}, i.e., relative performance of (outcross between population – self)/max. C) shows outbreeding depression is measured as RP_{WB}, i.e., relative performance of (outcross within population – outcross between population)/max. Species 1 = *M. ampliatus*, 2 = *M. dudleyi*, 3 = *M. floribundus*, 4 = *M. hymenophyllus*, 5 = *M. jungermannioides*, 6 = *M. patulus*, 7 = *M. washingtonensis*.

those between populations of the same species ($t = 3.92$, $df = 9$, $P < 0.01$). Within species outbreeding depression contrasts were more variable than higher taxonomic levels (Levene's test for equality of variances: $F = 5.970$, $P = 0.037$, Fig. 4-6.), supporting the notion of low phylogenetic inertia in this trait.

DISCUSSION

Inbreeding depression –

Inbreeding depression and outbreeding depression varied widely among populations in *M. moschatus* alliance studied here. This group of species is characterized by mating systems that ranged from obligately autogamous to primarily outcrossing ($t = 0.00 - 0.69$, see Chapter 2). Inbreeding depression was generally low for most populations (mean = 0.20), but ranged from around –0.13 to as high as 0.53. Thus, populations ranged from experiencing a modest fitness advantage to a roughly 50 % reduction in fitness upon selfing. In general, inbreeding depression values here were similar to overall means for angiosperms with this variation in selfing rates (selfing $\delta = 0.23$, outcrossing $\delta = 0.53$; Husband and Schemske 1996). Inbreeding depression was often present at the survival stage (see Chapter 3), indicating that the greenhouse environment was stressful to these progeny. Inbreeding depression values in greenhouse environments have been suggested to be underestimates of field values, because of a greater selective gradient in the more stressful field environment (Dudash 1990). Thus, it is difficult to estimate how closely inbreeding depression in this study would match those in the field, without conducting the field experiment. However, the greenhouse environment appeared moderately stressful to all populations (survivorship = ca. 60 %, Carlson unpublished data) and thus these values are probably reasonable approximations.

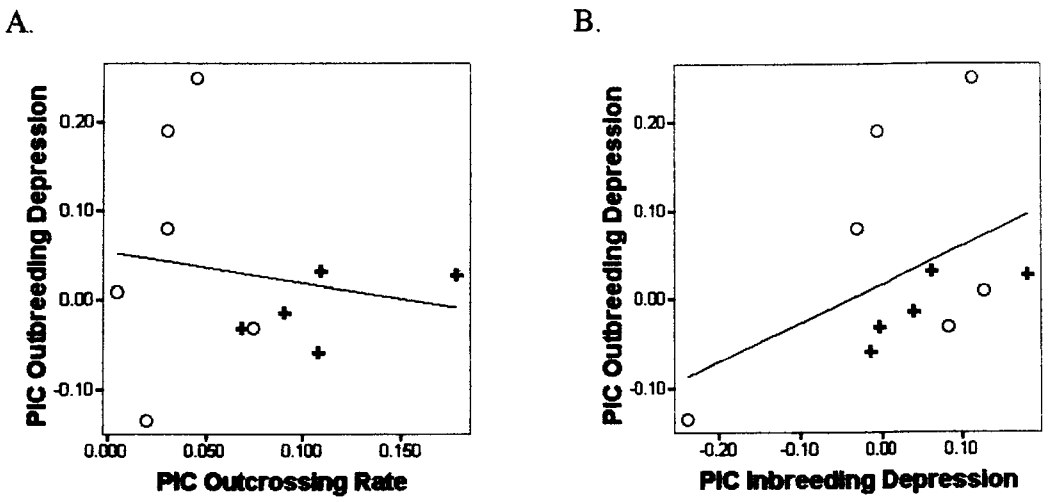

FIGURE 4-7. Phylogenetically independent contrasts between A) outbreeding depression and outcrossing rates and between B) outbreeding depression and inbreeding depression. Between population contrasts are indicated as circles, among species and higher level contrasts are indicated as plusses. No differences in outbreeding or inbreeding depression contrasts were present between populations of the same species relative to higher taxonomic levels (RP_{WB}: $t = 1.132$, $df = 9$, $P > 0.30$; RP_{WS}: $t = 0.043$, $df = 9$, $P > 0.50$). Within species outbreeding depression contrasts were more variable than higher taxonomic levels (Levene's test for equality of variances: $F = 5.970$, $P = 0.037$). Contrasts in outcrossing rates were significantly greater among higher levels relative to those between populations of the same species ($t = 3.92$, $df = 9$, $P < 0.01$).

Inbreeding depression was generally higher when measured as the fitness of between-population to selfed pollinations relative to the traditional within-population estimate. The fitness of between population relative to self crosses (RP_{BS}) were nearly always moderately to strongly positive, indicating that between population crosses were beneficial over selfing at least in the F1 generation. RP_{BS} values ranged from –0.05 to 0.53. Carr and Dudash (1996) observed similar results in a selfing and mixed-mating species in the *M. guttatus* species complex. They concluded that the selfing species possessed more non-segregating recessive deleterious alleles in populations than the more outcrossing species. However, the outcrossing species did appear to have some deleterious alleles for ovule production that were unique to some populations and have drifted to quite high frequencies (Carr and Dudash 1996). In my study, populations with high within-population outcross fitness relative to self fitness did not appear to have corresponding high between-population outcross fitness relative to self fitness. Thus, the two measures of self to outcross fitness, RP_{WS} and RP_{BS} did not appear to be coupled. This suggests that drift may be playing a large role even in mostly outcrossing populations.

It should be noted that sample sizes in this study did not appear to be large enough to detect relatively small levels of fitness differences. Confidence intervals tended to be large for all RP values (overall mean = ca. ±0.30), casting doubt on the precision of estimated means, used in the correlation analyses. Lack of precision in fitness parameters would result in a greater noise to signal ratio, generally weakening the strength of correlations (discussed below).

Genetics underlying inbreeding depression –

In general, there was a positive relationship between inbreeding depression and outcrossing rates in these populations (see Fig. 4-4), but this relationship was not particularly strong. The lack of a strong positive relationship may mean populations are not in equilibrium (see ***Phylogenetic effects*** – below). Alternatively, or in addition, many

ecological and genetic factors may mask the relationship (Uyenoyama et al. 1993). Pollen discounting (Holsinger et al. 1984), tradeoffs in offspring number (Uyenoyama et al. 1993), and resource costs of alternative reproductive strategies (Schemske 1978, Waller 1979) could influence levels of outcrossing independent of inbreeding depression. Further, the degree of inbreeding depression expressed has been shown to be a function of the particular environmental conditions (Dudash 1990, Waller submitted manuscript), so the strength of relationship between inbreeding depression and mating system will likewise be affected. In addition, for reasonable levels of dominance and mutation, inbreeding depression is expected to change very little as a function of mating system (Charlesworth et al. 1990, see Fig. 4-4). When dominance coefficients are greater than 0.02, the relationship between inbreeding depression and outcrossing rates is nearly linear, and there is little increase in inbreeding depression with increasing outcrossing rates (Johnston and Schoen 1996). For the *M. moschatus* alliance, it is difficult to estimate the levels of dominance and mutation in fitness contributing alleles since different populations with similar outcrossing rates had very different inbreeding depression levels and no populations were sampled that had nearly complete outcrossing. However, judging the fit of various parameter estimates to the data, the best estimate seems to be moderately low mutation rate ($U = 0.20$ mutations/sporophyte genome per generation) and low dominance ($h = 0.02$). Caution should taken in this extrapolation, as even closely related populations may have different genetic attributes of fitness alleles. In two species of *Amsinckia*, U was estimated as 0.83 and 0.32 mutations/sporophyte genome per generation and h was estimated as 0.32 and 0.10 (Johnston and Schoen 1995). For other species of *Mimulus*, inbreeding depression seems to be caused mostly by many genes of small effect with low dominance (Dudash and Carr 1998a, 1998b, Willis 1999).

In sum, this study found weak evidence for the expected positive relationship between inbreeding depression and outcrossing rates, supporting the partial dominance model of inbreeding depression. Because this relationship was not stronger, and similar results have been found elsewhere (e.g., Latta and Ritland 1994, Johnston and Schoen

1996), research should begin to focus on other factors likely responsible for mating system evolution.

Outbreeding depression/heterosis –

Outbreeding depression averaged –0.07 (range = –0.36 to 0.23). Thus, fitness was enhanced by 7 % on average by crossing between populations relative to within populations. With this experimental design it was not possible to explicitly evaluate the potential contribution of ecological versus genetic mechanisms of outbreeding depression. To test the ecological mechanism (i.e., local environmental adaptation) the parental populations need to be shown to be locally adapted and the F1 offspring need to be grown in the parental environments. Since all of these populations were grown in a common environment, the ecological contribution to outbreeding depression was eliminated and only the genetic contribution was expressed. In 14 populations, only one (*M. dudleyi* – 2), displayed moderately large (and marginally significant) levels of outbreeding depression, indicating that in this group, a coadapted gene environment was generally not disrupted in the F1s, nor was there evidence of underdominance for fitness loci. However if outbreeding depression is due to coadapted gene complexes, fitness decline should be greater in the F2 generation and subsequent generations, which was not measured here.

Most populations displayed moderate to considerable hybrid superiority upon outcrossing between population. In some cases, fitness increased 36 % over within population crosses. The F1 in populations of the annual legume, *Chamaecrista fasciculata*, often outperformed their parents (Fenster & Galloway 2000), as did the intertidal copepod, *Tigriopus californicus* (Edmands 1999), however subsequent generations experienced some loss of the originally observed F1 heterosis. Edmands (1999) suggested that the within chromosome breakup of parental gene combinations has both beneficial and negative effects. Fenster and Galloway (2000) indicate that population differentiation based on epistatic genetic divergence is molded by both selection and drift. Further, the F3 hybrid breakdown suggested that the epistatic

interactions were among linked loci. Additional generations are required here to evaluate the contributions of selection and drift on the observed F1 heterosis. However in the least, the F1 heterosis in these populations reveals population structuring and suggests that drift is a major contributor to population differentiation, as in Fenster and Galloway (2000).

It should be stressed that often one population in the reciprocal pairs appeared to be more fit than the other. The population hybrids often had higher fitness than one of the parental populations and lower fitness relative to the other population. Thus, the mean fitness of both within-population crosses relative to population-hybrid fitness was generally not different from zero, suggesting the fitness alleles behave additively. For one species however, *M. jungermannioides*, mean heterosis (0.18) was significantly greater than zero ($P < 0.009$). This species is highly clonal and it is possible that with limited recombination it suffers from the accumulation of slightly deleterious alleles, i.e., Muller's ratchet (see Muller 1932, Maynard Smith 1998)

Relationship between inbreeding depression and outbreeding depression –
I found no support for the hypothesis that mating system links inbreeding depression and outbreeding depression, and no evidence of a negative correlation between the two. While there was some evidence that inbreeding-depression levels are related to mating system (see Chapter 3), there was no relationship between outbreeding depression and mating system. In contrast to Fenster and Dudash's (1994) predictions, inbreeding depression and outbreeding depression tended to be positively correlated (only marginally significant in the phylogenetically naïve analysis, $R = 0.47$, $P < 0.10$). Populations with high levels of inbreeding depression were more likely to possess moderate levels of outbreeding depression. Populations with little or no inbreeding depression often expressed either no outbreeding depression, or moderate heterosis.

According to Fenster and Dudash (1994), inbreeding depression and outbreeding depression are predicted to correlate with outcrossing rates in opposite directions. This is because inbreeding depression should be greatest in more outcrossing populations due to

a higher genetic load of deleterious recessive alleles (Lande and Schemske 1985, Charlesworth and Charlesworth 1987). Conversely, high outbreeding depression is expected for species with high selfing rates, since greater linkage disequilibrium can be formed between fitness loci as recombination is more limited, allowing epistatic gene complexes to form rapidly. In this study, inbreeding depression was positively related to outcrossing rates as predicted. However, there was no relationship between outbreeding depression and mating system (outcrossing rates or anther-stigma separation).

One possible explanation for the positive relationship between inbreeding depression and outbreeding depression is that selfing populations become fixed for a number of mildly deleterious recessive alleles, so that outcrossing within population results in little fitness gain. However, crossing among populations results in increased fitness since each selfing population is fixed for different deleterious recessive alleles. Outcrossing populations are able to purge deleterious alleles through higher levels of recombination and therefore do not build up a large genetic load that can be masked by crossing to different populations. An additional segregating generation is needed to test whether deleterious alleles masked in the F1 are expressed in the F2.

It is possible that the causes of inbreeding depression and outbreeding depression are different for different taxa, or that taxa with different breeding systems have qualitatively different forms of inbreeding or outbreeding depression (e.g., Johnston and Schoen 1996). This seems unlikely, however, since much of the variation in crossing fitness is found within species, and therefore differences in gene action would presumably to take place between populations of the same species.

Phylogenetic effect –

Phylogenetic history was relatively unimportant in explaining the magnitude of both inbreeding and outbreeding depression relative to mating system and floral traits. Phylogenetic heritabilities (Lynch 1991) of mating system traits all approached 1.00 (Carlson unpublished manuscript), while inbreeding depression and outbreeding depression had much lower (near zero) phylogenetic heritabilites. Values that approach

zero indicate low phylogenetic inertia, in which trait inheritance from ancestry is minimal and values for taxa can be assumed independent (Lynch 1991, Martins 2001). Because of low phylogenetic inertia, correlation coefficients of phylogenetically corrected inbreeding and outbreeding values were all of similar magnitudes and directions to phylogenetically naïve correlations, and in some cases the relationships were even stronger after phylogenetic correction. These results indicate a decoupling in the coevolution of inbreeding depression, outbreeding depression, and mating system, disallowing equilibrium conditions to form. It is likely that other factors not investigated are more important in the evolution of these traits.

Conclusions and conservation implications –

In these 14 population in the *M. moschatus* alliance, inbreeding depression, outbreeding depression, and mating system varied broadly. Mating system tended to vary less between populations of the same species, while inbreeding depression and outbreeding depression varied as much with as between species. This disjunction between mating system and inbreeding and outbreeding depression variation suggests they are not in equilibrium and their coevolution is only loose and that other factors are important in their evolution. Contrary to expectation, inbreeding and outbreeding values were positively correlated with one another; populations that suffer from high inbreeding depression were also more likely to suffer from high outbreeding depression, while species with little or no inbreeding depression displayed little outbreeding depression or even heterosis. Inbreeding depression was positively correlated with outcrossing rate, but outbreeding depression did not appear to be related to mating system. Therefore, the development of coadapted gene complexes was either not any greater in selfing populations than in the more outcrossing populations, or there was a balancing effect of more inbred populations suffering from fixation of deleterious recessive alleles.

Most of the species in this study are remote, extremely geographically restricted and are found in relatively small population sizes (< 1,000 individuals). Further, six of seven species are annuals in arid locations that can be subject of population crashes in

years with dry, hot springs (personal observation). Thus, despite few anthropogenic threats, these populations are of great conservation concern (Carlson and Meinke 1994a, 1994b). Additionally, a number of these species suffered from high inbreeding depression in the greenhouse, indicating that if pollinator activity is reduced, severe fitness consequences may be the result. One or two generations of enforced inbreeding have been shown to negatively affect population persistence (Newman and Pilson 1997). Another conservation concern is the presence of outbreeding depression, which was marginally significant for a few populations, and was likely an underestimate since the local adaptation component was not included. Thus, for some populations, crossing between populations would result in a fitness decline. For most other populations, however, there appeared to be a modest fitness increase in the inter-population F1s. It should be stressed that this measure of fitness did not include local environmental adaptation and further, heterosis detected in the F1 is often lost in the F2 and future generations (Moll et al. 1965, Edmands 1999, Fenster and Galloway 2000). Therefore, mixing of populations to increase fitness is questionable in these populations. There is evidence of fixation of deleterious alleles within populations as a result of drift, and management efforts should be focused on maintaining high population sizes and high pollinator abundance.

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CONCLUSIONS

I explored the evolution of mating system in a group of phylogenetically related populations, focusing on the relationships among mating system characters and the influence of inbreeding depression as a primary selective factor.

In Chapter 1, I explored the evolutionary relationships within the *Mimulus moschatus* alliance, using morphological information and published molecular phylogenies. Four major clades were identified that have biogeographic associations. Further, this phylogenetic approach supported taxonomic recognition of *M. ampliatus* and *M. patulus*, two rare species, previously synonomized. Many morphological traits used in the analysis were very homoplastic, indicating they are highly evolvable in this group.

The primary question in this chapter was the direction of pollination-system evolution. Specifically, I asked if outcrossing lineages (large- and small-bee pollinated) give rise to selfers (not vice versa) and if selfing is an evolutionary "dead-end?" Ancestral-state reconstruction suggested that pollination systems appeared to shift from large- to small-bee pollinated, and occasional subsequent transitions to autogamous pollination systems. No unambiguous shifts from large-bee pollination to autogamy were detected; instead autogamy was generally evolved via transition through small-bee pollinated, small-flowered lineages. No cases of autogamy giving rise to more outcrossing pollination systems were detected, but one case of an autogamous ancestor giving rise to two autogamous species was indicated.

The secondary questions related to various biogeographic- and ecological-character evolution. First, I found evidence that widespread western North American species gave rise to multiple geographically restricted clades. Second, geographically restricted species in turn underwent limited speciation within their region, and no evidence of modern migration was present. Third, perennials on organically-rich substrates gave rise to annuals associated with organically-poor gravels, and subsequently

the perennial life-history was re-gained on two separate occasions. Last, seed dormancy evolution was a highly evolvable trait with at least five transitions occurring.

In Chapter 2, I investigated correlations among mating-system traits in the *M. moschatus* alliance, and its sister clade, the *M. guttatus* complex. These species ranged from highly inbred to moderately outcrossed, based on allozyme electrophoretic data. I found strong evidence that outcrossing rates were correlated with a suite of floral traits, traditionally used as mating-system proxies. Selfing taxa had small corollas with little anther-stigma separation and reduced pollen investment, while outcrossing taxa had large corollas with greater anther-stigma separation, and increased investment in pollen. Trait variation in this group fit the pattern of outcrossing taxa investing greater resources in male relative to female function. No tradeoff was detected between pollen number and volume. The high proportion of mixed mating taxa in this group adds support to the hypothesis that other genetic and ecological forces maintain intermediate mating systems.

In Chapter 3, I described the magnitude and timing of inbreeding depression in 14 populations (seven species) in the *M. moschatus* alliance and its relationship to mating system. I addressed three main points. 1) I found some support to the hypothesis of a positive association between "fitness" and "selfing" alleles among populations and species, i.e., inbreeding depression was weakly associated with anther-stigma separation, outcrossing rates, pollen/ovule ratio, corolla size, and autonomous seed set. However, the relationship was not strong, and selective forces other than inbreeding depression are likely important in the evolution of plant mating systems. 2) Taxa inherited a significant portion of both mating-system traits and to a much lesser degree, inbreeding depression from their ancestors. Mating system (both outcrossing rates and floral morphology) appeared to be more evolutionary constrained than is inbreeding depression. I suggested that the occurrence of random mutations of deleterious alleles of large effect may be largely responsible for the current distribution of inbreeding-depression levels in this group. 3) There was no clear sequence of evolution of mating system and inbreeding depression, and no evidence that inbreeding depression promotes the evolution of outcrossing, nor that selfing reduces inbreeding depression. Last, outcrossing rates were

found to be substantially more evolvable than floral traits, supporting the notion that ecological factors can be as, or more important than morphological features in determining mating system evolution.

In the final chapter, I tested the relationship among inbreeding depression, outbreeding depression, and mating system in 14 population in the *M. moschatus* alliance. Inbreeding depression and outbreeding depression appeared to fluctuate on a smaller evolutionary-time scale than did mating system. This disjunction between mating system and inbreeding and outbreeding depression variation suggests they are not in equilibrium, their coevolution is only loose, and that other factors are important in their evolution. Contrary to expectations, inbreeding and outbreeding values were weakly, positively correlated with one another. Populations that suffered from high inbreeding depression were also more likely to suffer from high outbreeding depression, while species with little or no inbreeding depression showed little outbreeding depression or even heterosis. Inbreeding depression was positively correlated with outcrossing rate, but outbreeding depression did not appear to be related to mating system. Therefore, the development of coadapted gene complexes was either not any greater in selfing populations than in the more outcrossing populations, or there was a balancing effect of more inbred populations suffering from fixation of deleterious recessive alleles.

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as a result of drift, and management efforts should be focused on maintaining high population sizes and high pollinator abundance.

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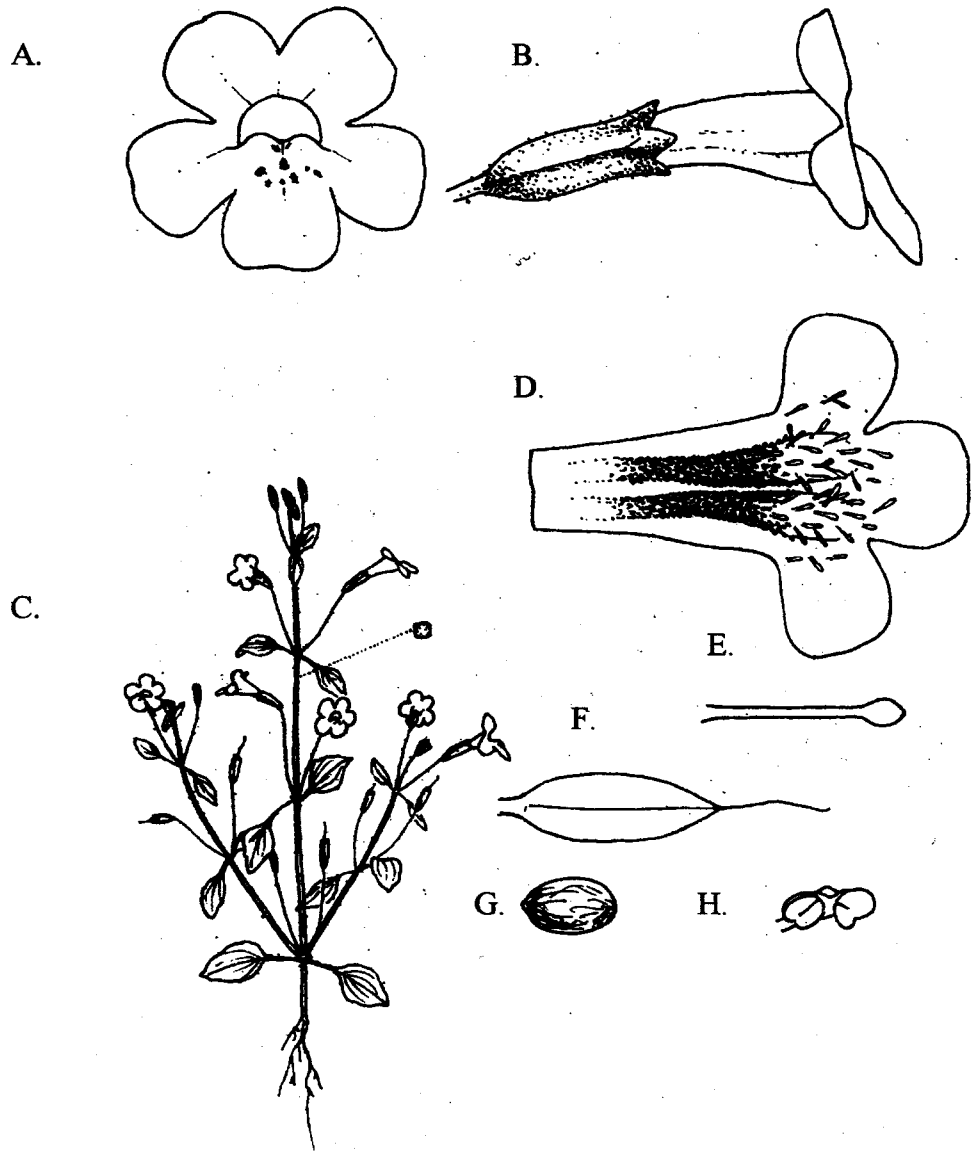
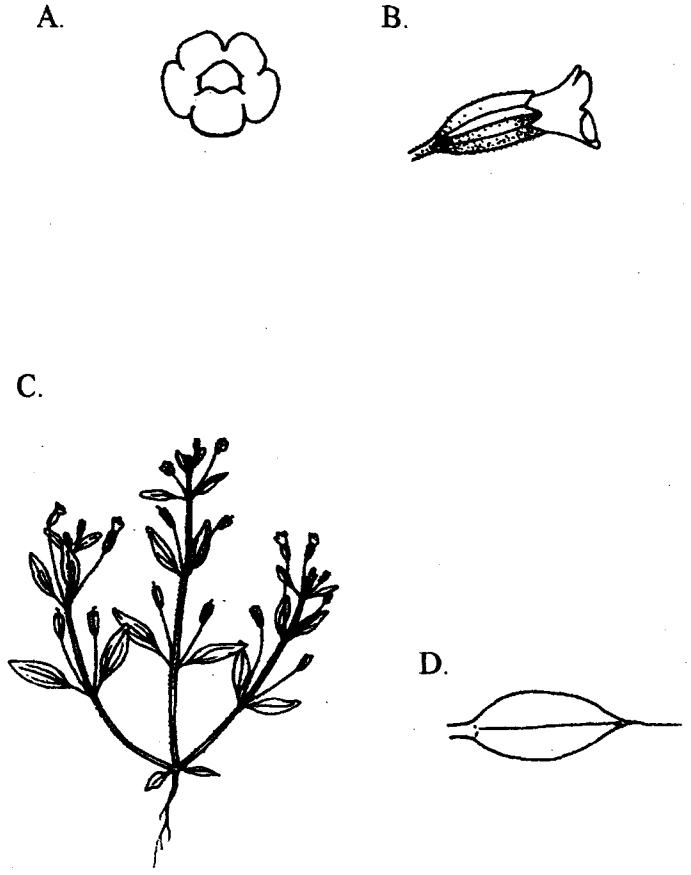
APPENDIX I

ILLUSTRATIONS OF THE *MIMULUS MOSCHATUS* ALLIANCE

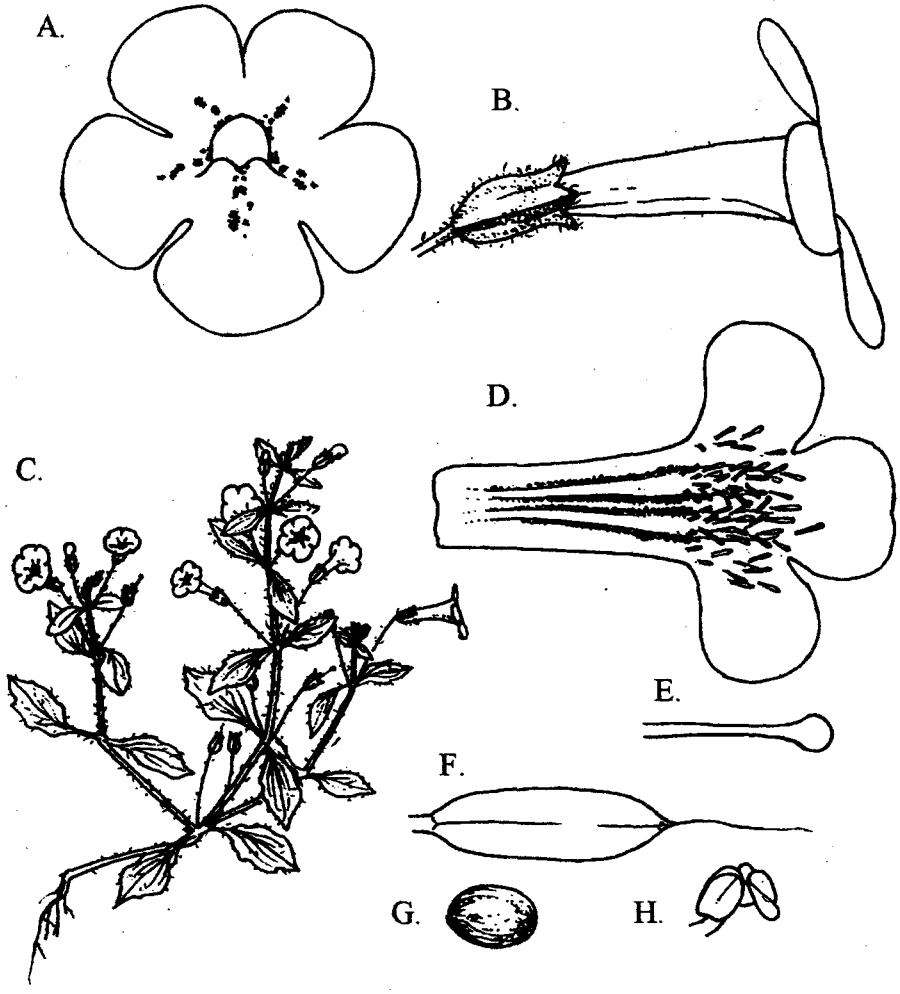
Mimulus ampliatus. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D. = longitudinal cross section of corolla, E.= style and stigma, F. = capsule in fruit, G. = seed, H. = anther.



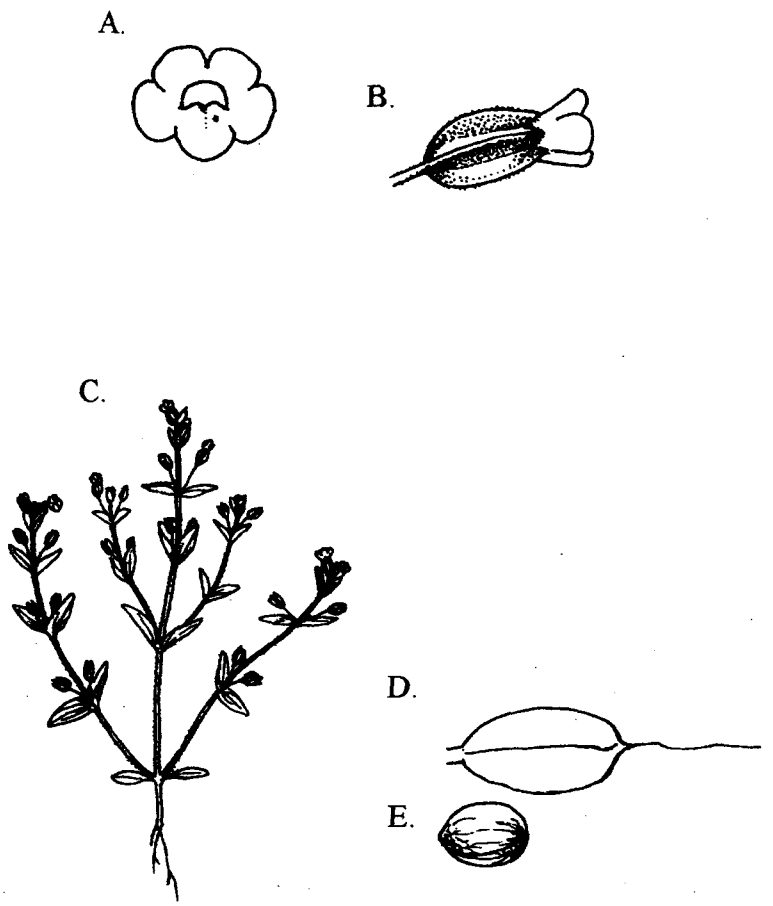
Mimulus breviflorus. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D. = capsule in fruit.



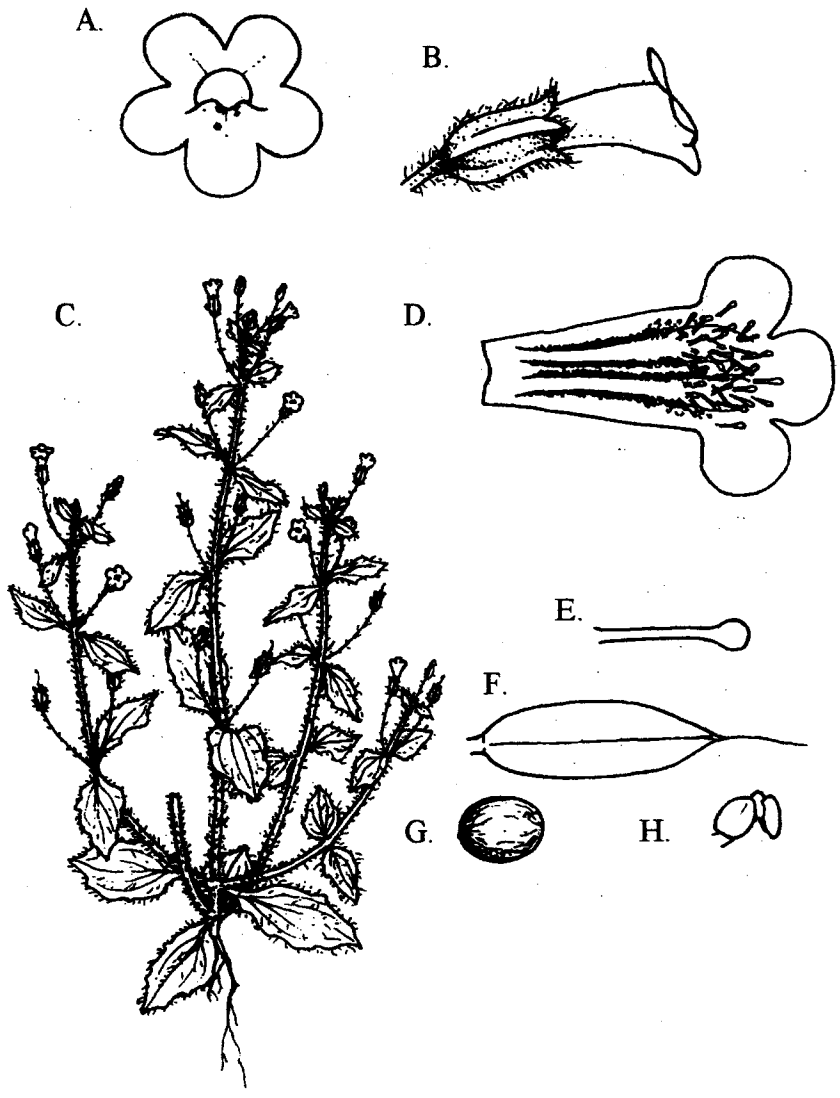
Mimulus dudleyi. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D. = longitudinal cross section of corolla, E. = style and stigma, F. = capsule in fruit, G. = seed, H. = anther.



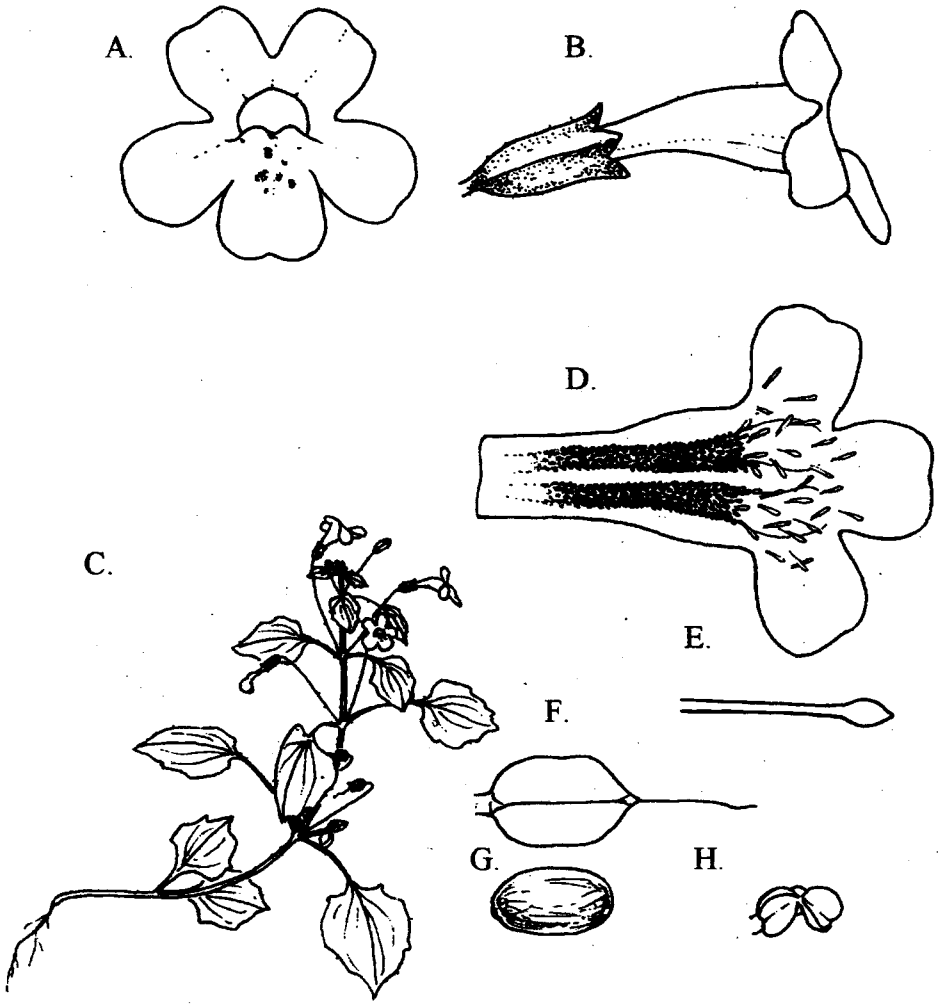
Mimulus evanescens. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D. = capsule in fruit, E = seed.



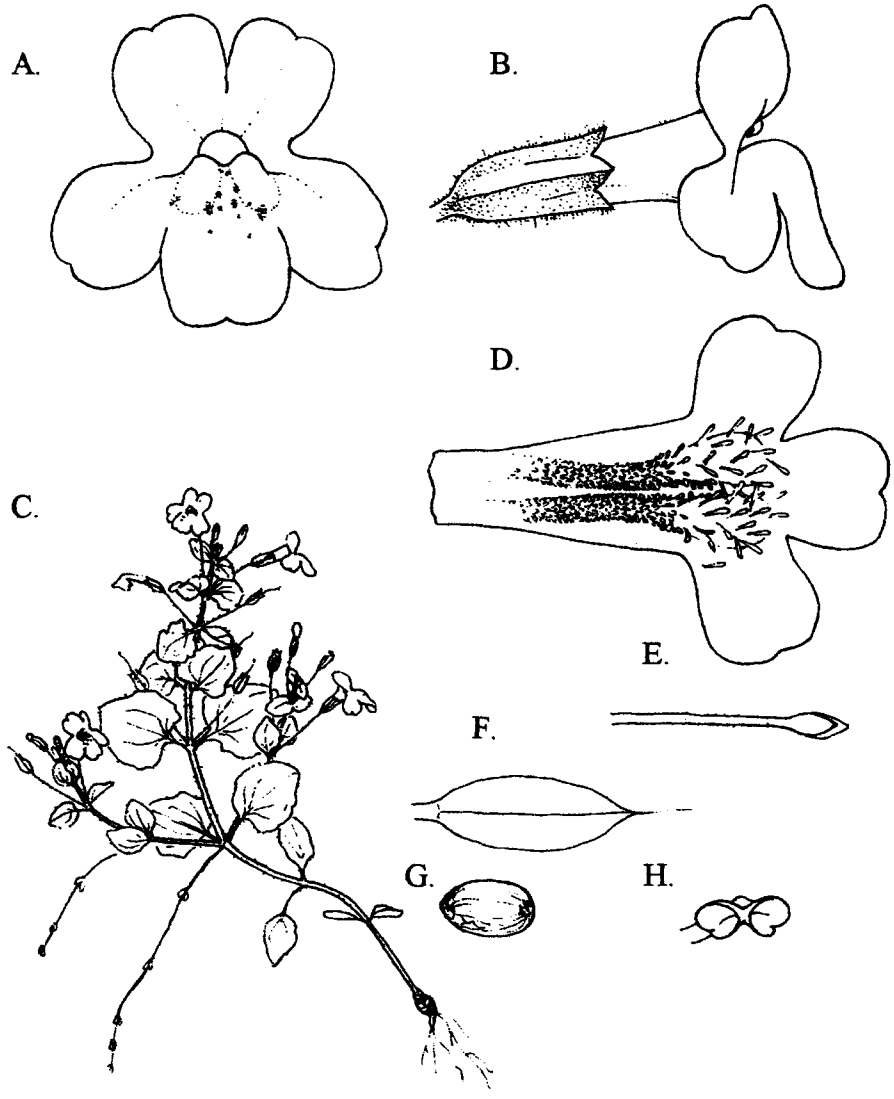
Mimulus floribundus. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D. = longitudinal cross section of corolla, E. = style and stigma, F. = capsule in fruit, G. = seed, H. = anther.



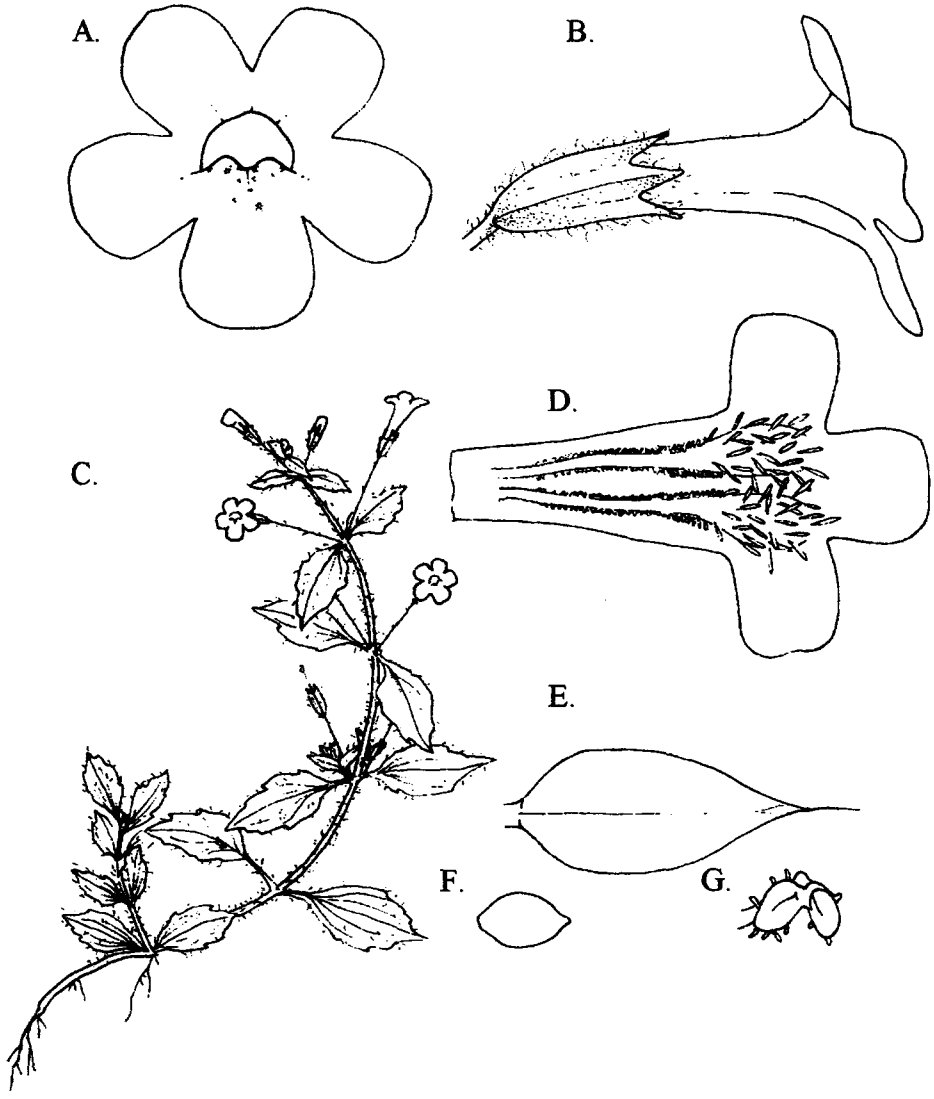
Mimulus hymenophyllus. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D. = longitudinal cross section of corolla, E. = style and stigma, F. = capsule in fruit, G. = seed, H. = anther.



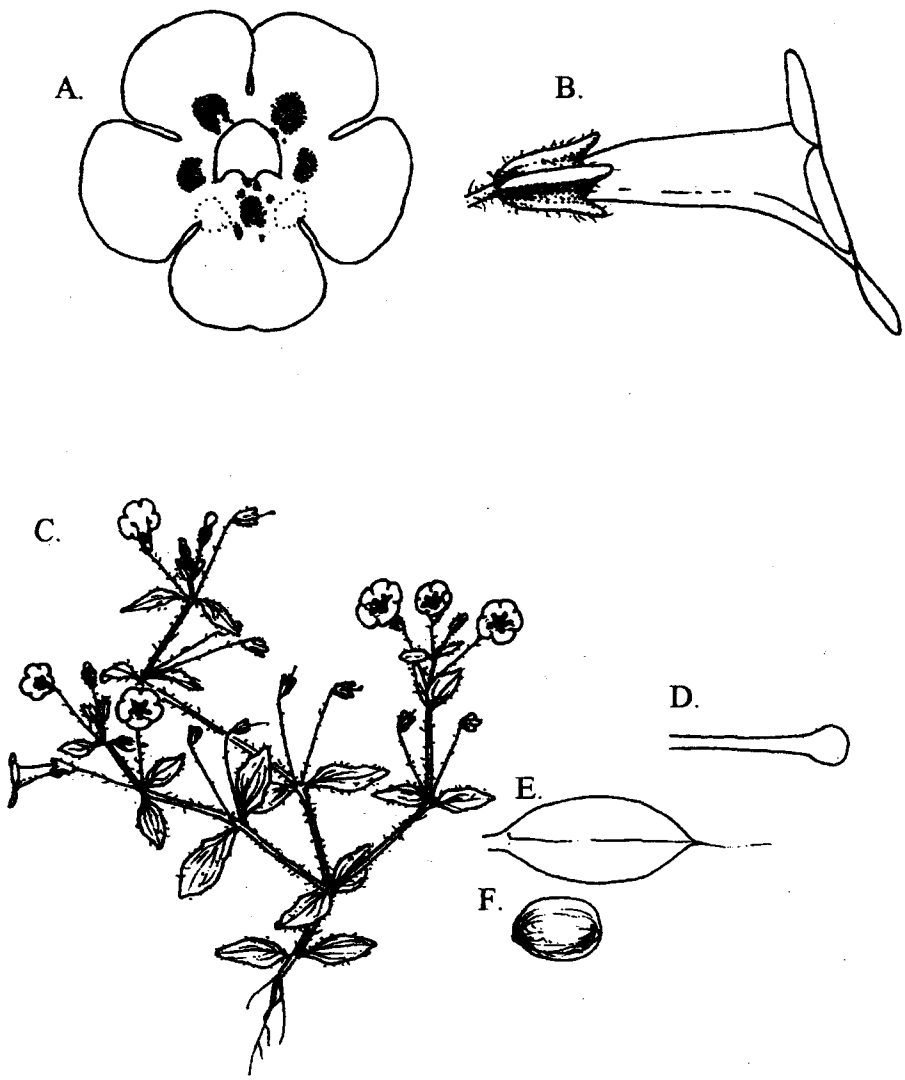
Mimulus jungermannioides. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D. = longitudinal cross section of corolla, E. = style and stigma, F. = capsule in fruit, G. = seed, H. = anther.



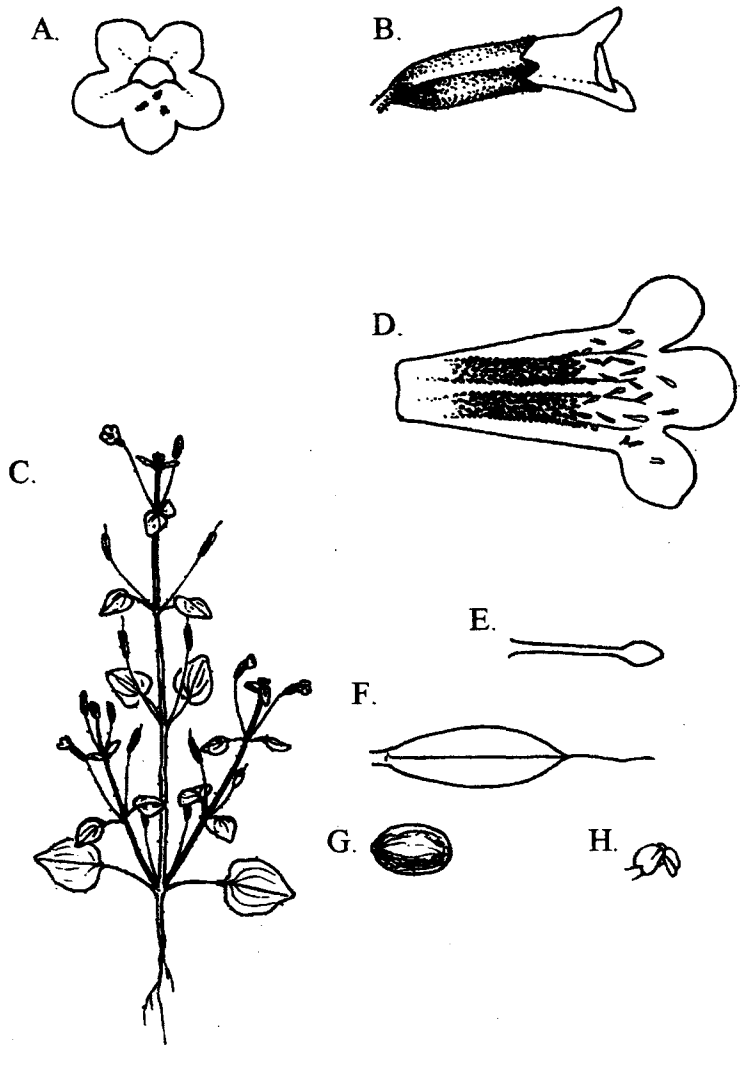
Mimulus moschatus. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D. = longitudinal cross section of corolla, E. = capsule in fruit, F. = seed, G. = anther.



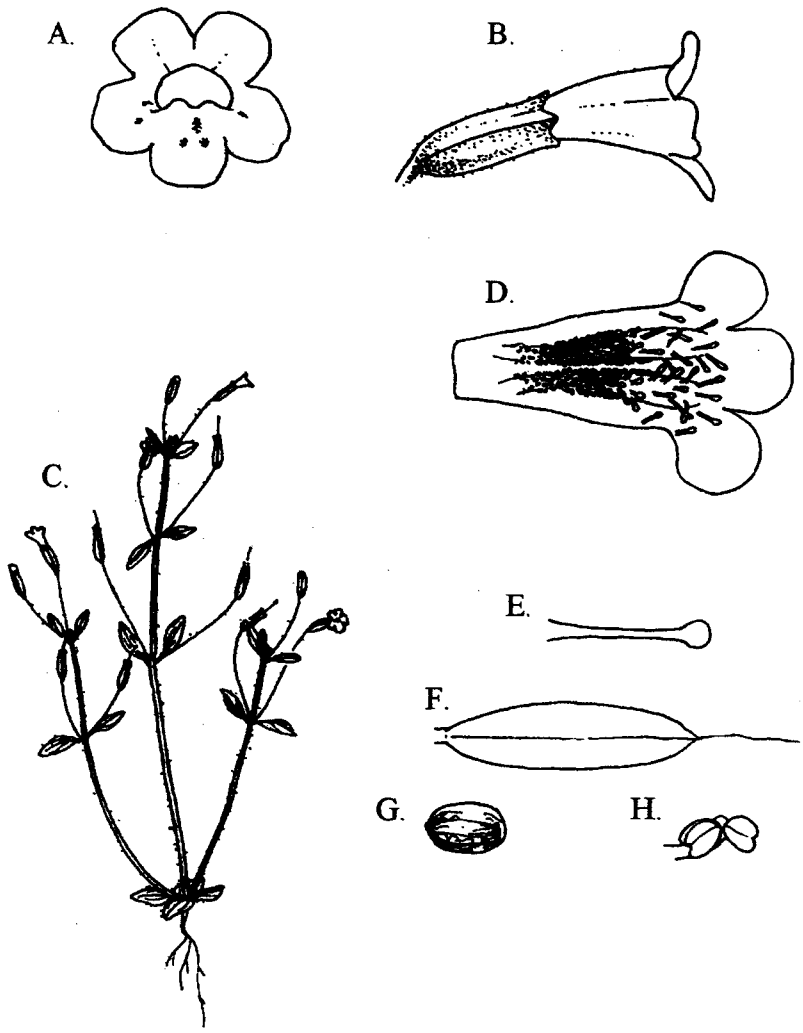
Mimulus norisii. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D. = style and stigma, E. = capsule in fruit, F. = seed.



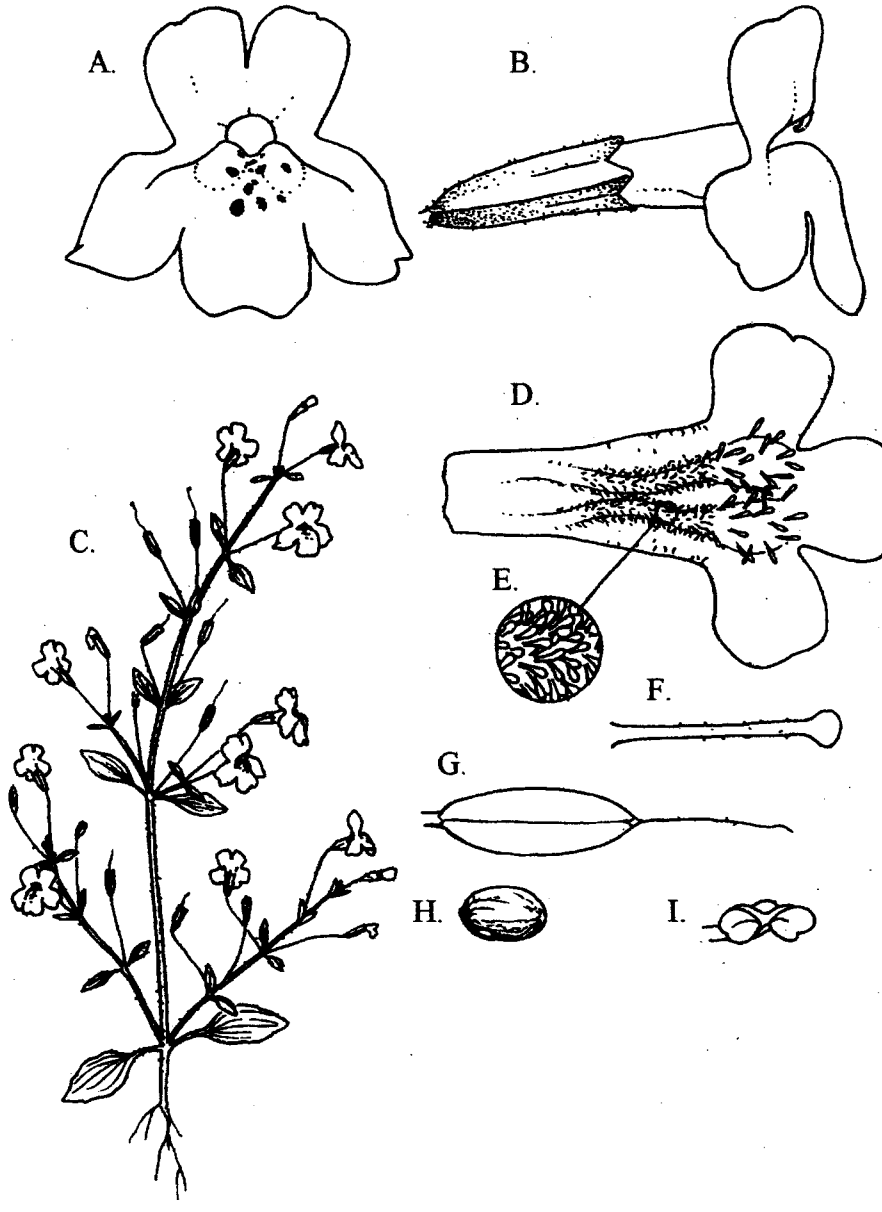
Mimulus patulus. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D. = longitudinal cross section of corolla, E. = style and stigma, F. = capsule in fruit, G. = seed, H. = anther.



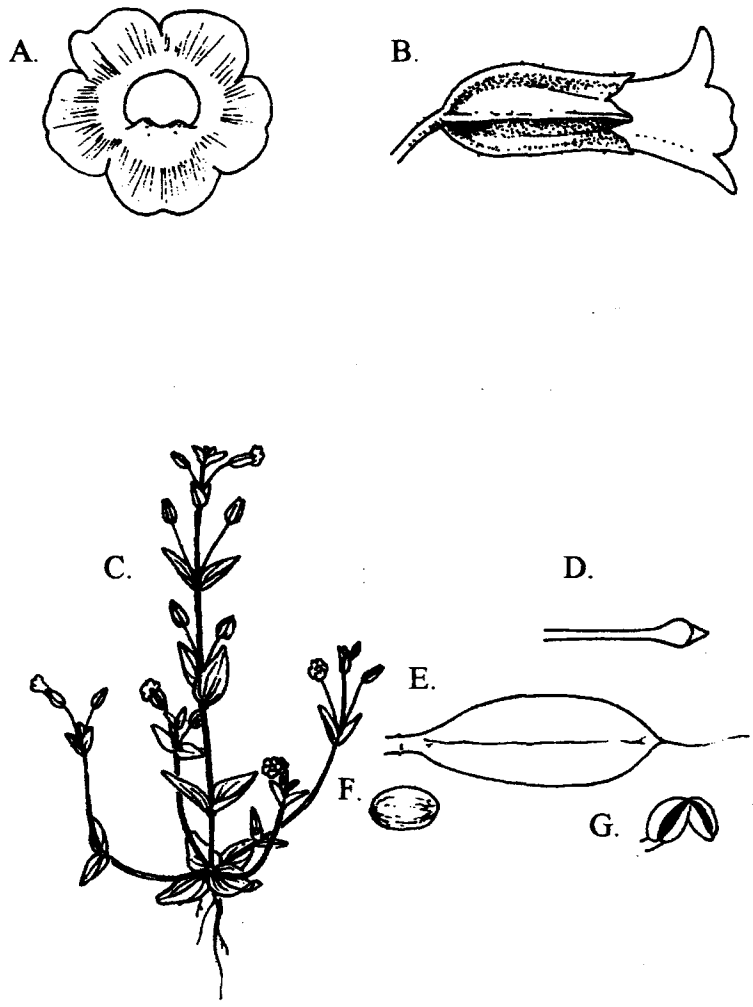
Mimulus pulsiferae. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D. = longitudinal cross section of corolla, E. = style and stigma, F. = capsule in fruit, G. = seed, H. = anther.



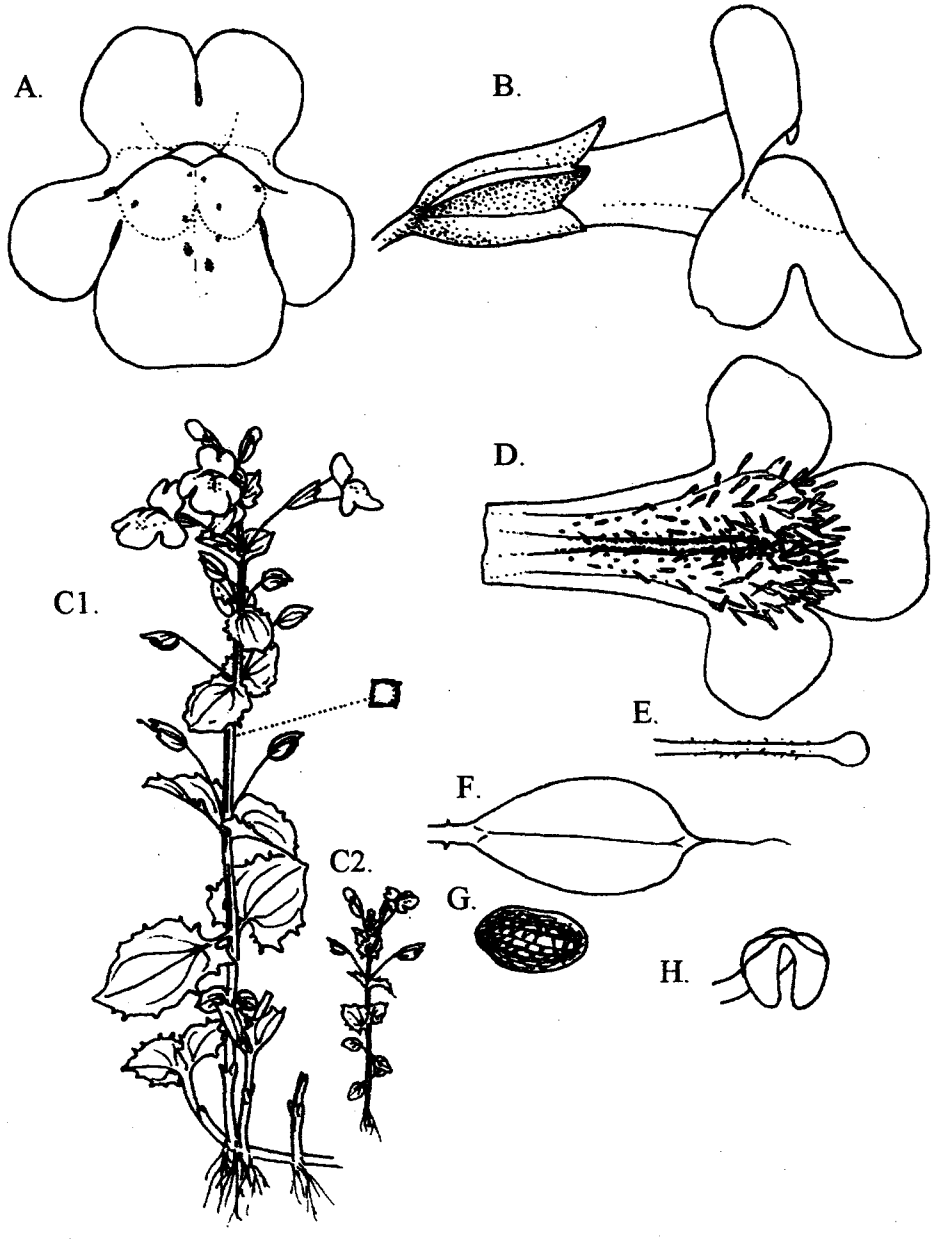
Mimulus washingtonensis. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D. = longitudinal cross section of corolla, E. = close-up view of micro-clavate palate pubescence, F = style and stigma, G. = capsule in fruit, H. = seed, I. = anther.



Mimulus latidens, nearest outgroup to the *M. moschatus* alliance. A. = Corolla (front), B. = corolla/calyx (side), C. = plant, D = style and stigma, E. = capsule in fruit, F. = seed, G. = anther.



Mimulus guttatus, section Simiolus. A. = Corolla (front), B. = corolla/calyx (side), C1. = large-flowered, perennial form, showing square stem in cross-section, C2. = small-flowered, annual form, D. = longitudinal cross section of corolla, E. = pubescent style and obovate stigma, F. = capsule in fruit, G. = seed with obvious reticulations, H. = anther showing incomplete dehiscence.



APPENDIX II

FLORAL ARCHITECTURE

TABLE A-II-1. Corolla morphology in 14 populations (seven species) in the *M. moschatus* alliance. All measurements are given in mm. Means are followed by standard errors.

Species-Population	Corolla height	Corolla width	Corolla length	Tube depth	Aperture height	Aperture width
M. ampliatus-1	11.81 ± 0.40 (n = 22)	13.43 ± 0.36 (n = 22)	16.88 ± 0.34 (n = 22)	15.10± 0.30 (n = 22)	4.42 ± 0.12 (n = 22)	5.33 ± 0.13 (n = 22)
M. ampliatus-2	10.97 ± 0.41 (n =23)	12.58 ± 0.35 (n = 23)	15.50 ± 0.28 (n = 23)	14.00± 0.25 (n = 23)	3.90 ± 0.14 (n = 23)	4.93 ± 0.16 (n = 23)
M. dudleyi-1	13.51 ± 0.50 (n = 18)	14.14 ± 0.56 (n = 18)	15.01 ± 0.31 (n = 18)	14.63 ± 0.33 (n = 18)	4.28 ± 0.11 (n = 18)	4.18 ± 0.19 (n = 18)
M. dudleyi-2	13.71 ± 0.56 (n = 18)	14.59 ± 0.58 (n = 18)	15.49 ± 0.40 (n = 18)	14.95 ± 0.40 (n = 18)	4.30 ± 0.12 (n = 18)	4.35 ± 0.15 (n = 18)
M. floribundus-1	7.92 ± 0.31 (n = 17)	9.04 ± 0.35 (n = 17)	11.62 ± 0.17 (n = 17)	10.66 ± 0.22 (n = 17)	3.04 ± 0.10 (n = 17)	3.53 ± 0.09 (n = 17)
M. floribundus-2	8.19 ± 0.32 (n = 17)	9.35 ± 0.32 (n = 17)	11.87 ± 0.19 (n = 17)	10.92 ± 0.17 (n = 17)	3.31 ± 0.11 (n = 17)	3.60 ± 0.14 (n = 17)
M. hymenophyllus-1	10.57 ± 0.33 (n = 23)	11.70 ± 0.34 (n = 23)	16.07 ± 0.27 (n = 23)	14.28 ± 0.27 (n = 23)	3.91 ± 0.08 (n = 23)	4.56 ± 0.11 (n = 23)
M. hymenophyllus-2	9.83 ± 0.31 (n = 22)	10.88 ± 0.31 (n = 22)	14.27 ± 0.33 (n = 22)	12.58 ± 0.24 (n = 22)	3.76 ± 0.11 (n = 22)	4.48 ± 0.12 (n = 22)
M. jungermannioides-1	13.87 ± 0.43 (n = 17)	14.48 ± 0.42 (n = 17)	15.81 ± 0.26 (n = 17)	14.06 ± 0.40 (n = 17)	4.13 ± 0.10 (n = 17)	4.21 ± 0.13 (n = 17)
M. jungermannioides-2	14.08 ± 0.31 (n = 18)	15.32 ± 0.24 (n = 18)	16.75 ± 0.28 (n = 18)	14.61 ± 0.20 (n = 18)	3.98 ± 0.08 (n = 18)	4.29 ± 0.11 (n = 18)
M. patulus-1	4.54 ± 0.19 (n = 21)	6.14 ± 0.45 (n = 21)	9.51 ± 0.23 (n = 21)	8.65 ± 0.28 (n = 21)	2.17 ± 0.07 (n = 21)	2.47± 0.12 (n = 21)
M. patulus-2	4.96 ± 0.19 (n = 23)	5.97 ± 0.21 (n = 23)	9.22 ± 0.15 (n = 23)	8.25 ± 0.14 (n = 23)	2.19 ± 0.06 (n = 23)	2.48 ± 0.08 (n = 23)
M. washingtonensis-1	12.93 ± 0.31 (n = 21)	13.98 ± 0.32 (n = 21)	14.22 ± 0.21 (n = 21)	11.80 ± 0.18 (n = 21)	3.44 ± 0.07 (n = 21)	3.57 ± 0.13 (n = 21)
M. washingtonensis-2	13.20 ± 0.43 (n = 21)	14.81 ± 0.48 (n = 21)	14.27 ± 0.24 (n = 21)	11.54 ± 0.22 (n = 21)	3.85 ± 0.12 (n = 21)	3.70 ± 0.11 (n = 21)

TABLE A-II-2. Stigma and anther measurements in 14 populations (seven species) in the *M. moschatus* alliance. All measurements are given in mm. Means are followed by standard errors.

Species-Population	Stigma length	Stigma position	Pistil length	Proximal anther position	Distal anther position	Anther-stigma separation
M. ampliatus-1	1.78 ± 0.05 (n = 13)	13.89 ± 0.23 (n = 22)	14.84 ± 0.30 (n = 18)	12.12 ± 0.22 (n = 22)	13.28 ± 0.22 (n = 22)	0.61 ± 0.09 (n = 22)
M. ampliatus-2	1.65 ± 0.05 (n = 11)	13.21 ± 0.21 (n = 23)	14.00 ± 0.27 (n = 21)	11.42 ± 0.19 (n = 23)	12.51 ± 0.20 (n = 23)	0.70 ± 0.09 (n = 23)
M. dudleyi-1	1.66 ± 0.09 (n = 4)	9.02 ± 0.17 (n = 18)	10.03 ± 0.17 (n = 18)	6.65 ± 0.13 (n = 18)	7.93 ± 0.12 (n = 18)	1.08 ± 0.09 (n = 18)
M. dudleyi-2	1.75 ± 0.09 (n = 6)	9.24 ± 0.16 (n = 18)	10.27 ± 0.17 (n = 18)	6.95 ± 0.14 (n = 18)	8.11 ± 0.14 (n = 18)	1.12 ± 0.08 (n = 18)
M. floribundus-1	1.15 ± 0.05 (n = 2)	8.76 ± 0.14 (n = 17)	9.46 ± 0.17 (n = 17)	8.31 ± 0.15 (n = 17)	8.92 ± 0.15 (n = 17)	-0.16 ± 0.04 (n = 17)
M. floribundus-2	1.00 – (n = 1)	9.05 ± 0.12 (n = 17)	9.72 ± 0.15 (n = 17)	8.55 ± 0.11 (n = 17)	9.17 ± 0.12 (n = 17)	-0.13 ± 0.06 (n = 17)
M. hymenophyllus-1	1.51 ± 0.05 (n = 10)	13.20 ± 0.21 (n = 23)	13.89 ± 0.25 (n = 20)	12.05 ± 0.18 (n = 23)	13.07 ± 0.20 (n = 23)	0.13 ± 0.05 (n = 23)
M. hymenophyllus-2	1.43 ± 0.07 (n = 9)	11.83 ± 0.22 (n = 22)	12.58 ± 0.20 (n = 19)	10.91 ± 0.17 (n = 22)	11.86 ± 0.19 (n = 22)	-0.02 ± 0.08 (n = 22)
M. jungermannioides-1	1.67 ± 0.08 (n = 6)	14.12 ± 0.19 (n = 17)	14.94 ± 0.18 (n = 17)	12.20 ± 0.21 (n = 17)	13.71 ± 0.23 (n = 17)	0.41 ± 0.06 (n = 17)
M. jungermannioides-2	1.71 ± 0.08 (n = 5)	14.87 ± 0.16 (n = 21)	15.90 ± 0.17 (n = 18)	12.56 ± 0.19 (n = 18)	14.45 ± 0.17 (n = 21)	0.41 ± 0.09 (n = 21)
M. patulus-1	1.11 ± 0.06 (n = 7)	7.81 ± 0.37 (n = 21)	8.00 ± 0.16 (n = 18)	7.49 ± 0.27 (n = 21)	8.00 ± 0.29 (n = 21)	-0.25 ± 0.03 (n = 21)
M. patulus-2	0.09 ± 0.04 (n = 13)	7.46 ± 0.10 (n = 23)	8.00 ± 0.09 (n = 20)	7.03 ± 0.14 (n = 23)	7.62 ± 0.09 (n = 23)	-0.17 ± 0.03 (n = 23)
M. washingtonensis-1	1.59 ± 0.05 (n = 13)	12.79 ± 0.17 (n = 21)	13.58 ± 0.19 (n = 21)	10.68 ± 0.16 (n = 21)	11.96 ± 0.18 (n = 21)	0.88 ± 0.12 (n = 21)
M. washingtonensis-2	1.73 ± 0.07 (n = 11)	12.79 ± 0.21 (n = 21)	13.47 ± 0.29 (n = 21)	11.14 ± 0.36 (n = 21)	12.03 ± 0.24 (n = 21)	0.77 ± 0.13 (n = 21)

APPENDIX III

ALLOZYME RECIPES

***Extraction buffer*:**

0.01 g EDTA

0.019 g KCl

0.05 g $MgCl_2$

1.0 g PVP

25 ml 0.1 M Tris-HCl, pH 7.5

- Just before grinding add 1 drop of 2-mercaptoethanol to 5 ml grinding buffer (covered and kept on ice).
- At 0° – 4° C, grind 2 cm^2 of fresh, young tissue with 1-2 drops grinding buffer. Blot ground tissue/buffer with 3 mm x 10 mm Whatman 3 mm CHR paper wicks. Samples (wicks blotted with ground tissue/buffer) can be stored at -80° C until used.

***Electrode buffers*:**

Morpholine-Citrate (0.04 M citric acid titrated with morpholine)

7.69 g citric acid (anhydrous)

1.0 L DH_2O

titrated to pH 6.1 with N-(3-amminopropyl) morpholine

Electrode buffer 5 (0.223 M Tris, 0.069 M citric acid)

27.0 g Tris

13.1 g citric acid (anhydrous)

1.0 L DH_2O

pH 7.2 at 22° C

Electrode buffer 6 (0.100 M NaOH, 0.300 M boric acid)

4.0 g NaOH

18.55 g boric acid

1.0 L DH_2O

pH 8.6 at 22 °C

Gel buffers:

Gel buffer Morpholine-Citrate

36 ml of Morpholine-Citrate electrode buffer to 1 L DH_2O

Gel buffer 5 (0.009 M Tris, 0.003 M citric acid)

1.089 g Tris

0.576 g citric acid (anhydrous)

1 L DH_2O

pH 7.2 at 22 °C

or dilute 35 ml electrode buffer 5 to 1 L DH_2O

Gel buffer 6 (0.015 M Tris, 0.004 M citric acid

1.84 g Tris

0.69 g citric acid (anhydrous)

1 L DH_2O

pH 7.8 at 22 °C

Gel: (11.7 % starch)

35 g potato starch

300 ml gel buffer

- Swirl constantly over a flame, until the mixture comes to a rolling boil. De-gas with an aspirator, until only large bubbles are present, and pour starch into an acrylic mold. After cooling to room temperature cover the starch with cling-wrap and let it sit for 12 hrs.
- Gels are cooled to 4 °C before the samples are applied. A slit, serving as the origin, is cut on the cathodal edge perpendicular to the gel surface 3 cm from the edge of gel, into which the blotted wicks are applied. Chilled electrode buffer trays are filled with appropriate buffers. Gels are run at 4 °C
- Morpholine citrate run at 40 mAmps, (150-200 V) for 5 hrs.
- Buffer system 5 run at 40 mAmps (100-140 V) for 5 hrs.
- Buffer system 6 at 35 mAmps (110 V) for 5 hrs.

Stains:

Acid phosphatase (ACP)

50 ml - 50 mM Na-acetate buffer, pH 5.0

50 mg – Na-α-napthyl acid phosphate

1 ml – 1 M $MgCl_2$

50 mg – Fast garnet GBG salt

Alcohol dehydrogenase (ADH)

50 ml – mM Tris-HCl, pH 8.0

0.2 ml – ethanol

1 ml – NAD (5 mg/ml)

1 ml – MTT (5 mg/ml)

1 ml – PMS (5 mg/ml)

Aspartate aminotransferase (AAT)

50 ml - AAT substrate solution:

100 ml – DH_20

0.037 g – α-ketoglutaric acid

0.134 g – L-aspartic acid

0.50 g – PVP-40

0.050 g – EDTA, NA_2 salt

1.42 g – sodium phosphate, dibasic

50 mg – Fast blue BB salt

Diaphorase (DIA)

25 ml – 200 mM Tris-200 mM maleate pH 3.7

10 ml – 0.2 N NaOH

15 ml - DH_20

1 ml - $MgCl_2$

25 mg – α-N-Benzoly-DL-β-Naphtylamide-HCl

2 ml – N, N-Dimethylformamide

20 mg – Fast black K salt

Glucose-6-phosphate dehydrogenase (G6PDH)

50 ml – Tris-HCl, pH 8.0

1 ml – $MgCl_2$ (50 mg/ml)

50 mg – Glucose-6-phosphate, Na2 salt

1 ml – NADP (5 mg/ml)

1 ml – MTT (5 mg/ml)

1 ml – PMS (5 mg/ml)

Isocitrate dehydrogenase (IDH)

50 ml – 50 mM Tris-HCl, pH 8.0

1.5 ml – $MgCl_2$ (50 mg/ml)

80 mg – Isocitric acid, Na_3 salt

10 mg – NAPD (dry)

1 ml – MTT (5 mg/ml)

1 ml – PMS (5 mg/ml)

Malate dehydrogenase (MDH)

50 ml – 50 mM Tris-HCl, pH 8.5

150 mg – Malic acid

1 ml – NAD (5 mg/ml)

1 ml – MTT (5 mg/ml)

1 ml – PMS (5 mg/ml)

Phosphoglucose isomerase (PGI)

50 ml – 50 mM Tris-HCl, pH 8.0

20 mg – Fructose-6-phosphate, Na_2 salt

20 units – Glucose-6-phophate dehydrogenase (NAD)

1 ml – NAD (5 mg/ml)

1 ml – MTT (5 mg/ml)

1 ml – PMS (5 mg/ml)

Phosphoglucomutase (PGM)

50 ml – 50 mM Tris-HCl, pH 8.5

150 mg – Glucose-1-phosphate, Na_2 salt

20 units – Glucose-6-phophate dehydrogenase (NAD)

1 ml – NAD (5 mg/ml)

1 ml – MTT (5 mg/ml)

1 ml – PMS (5 mg/ml)

Phosphogluconate dehydrogenase (6PGD)

50 ml – 50 mM Tris-HCl, pH 8.0

20 mg – 6-Phosphogluconic acid, Na_2 salt

1 ml – $MgCl_2$ (50 mg/ml)

1 ml – NADP (5 mg/ml)

1 ml – MTT (5 mg/ml)

1 ml – PMS (5 mg/ml)

Shikimate dehydrogenase (SKDH)

50 ml – 50 mM Tris-HCl, pH 8.5

50 mg – Shikimic acid

1 ml – NADP (5 mg/ml)

1 ml – MTT (5 mg/ml)

1 ml – PMS (5 mg/ml)

Triose-phosphate isomerase (TPI)

50 ml – 50 mM Tris-HCl, pH 8.0

75 mg – arsenic acid, Na_2 salt

10 mg – Dihydroxyacetone phosphate

300 units – Glyceraldehyde-3-phosphate dehydrogenase

1 ml – NAD (5 mg/ml)

1 ml – MTT (5 mg/ml)

1 ml – PMS (5 mg/ml)

- In all cases, combine ingredients and pour over gel slices. Incubate in the dark at 37 °C for one hour, or more, until bands are developed. ADH is covered with cling-wrap to prevent escape of volatized ethanol.